THE CO-REGULATION OF THE MUCUS ASSOCIATED MOLECULES INTELECTIN, RESISTIN LIKE MOLECULE BETA AND BETA GALACTOSIDE ALPHA 2-3 SIALYLTRANSFERASE IN A T HELPER CELL TYPE 2 RESPONSE

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DECLARATION

I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Edinburgh

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ABSTRACT

Increased mucin production and goblet cell hyperplasia are common features of parasitic and allergic disease in man and animals. A protective role for mucus has been suggested in gastro-intestinal parasitic infections in rodents. The mucus associated molecules intelectin (ITLN), resistin like molecule beta (RELMβ) and beta galactoside alpha 2-3 sialyltransferase (SIAT4C) are upregulated in nematode infections known to induce a typical T helper cell type 2 (Th2) response in mice. In the present work, it was hypothesised that these three mucus-associated molecules were co-regulated by Th2 cytokines and that their upregulation was part of a typical anti-parasite response in other species. Sheep were chosen as a model because of the economic importance of both respiratory and gastrointestinal tract parasitic infections in sheep.

Culture of a human colonic carcimoma cell line with either interleukin 4 (IL-4) or IL-13 confirmed the upregulation of ITLN and RELMβ in a Th2 environment but failed to show co-regulation with SIAT4C. Of the Th1 or Th2 cytokines examined only IFNγ was found to have a significant effect on expression of SIAT4C transcript. ITLN transcript and protein were demonstrated in sheep tissue and furthermore three different ITLNs (sITLN1, sITLN2, sITLN3) which had a differential tissue distribution were cloned and sequenced. SIAT4C was shown to be widely expressed in sheep tissues and the full sequence was deduced from expressed sequence tags, and confirmed. There was no evidence of expression of RELMβ in sheep tissues examined.

sITLN transcripts were shown to be upregulated in response to IL-4 in an *ex vivo* sheep tracheal explant culture model whilst sheep (s) SIAT4C was significantly downregulated in the same model. However, despite differential regulation by IL-4 *ex vivo*, in a sheep model of infection with the abomasal nematode, *Teladorsagia circumcincta*, known to induce a Th2 biased response, sITLN transcripts and protein and sSIAT4C transcript were upregulated in response to a challenge infection.

Furthermore, sITLN1 and sITLN2 were shown to upregulate at an earlier time point post challenge in previously infected (immune) compared to naïve yearling sheep and lambs and significant upregulation of sSIAT4C transcript was seen in previously infected challenged but not naïve challenged sheep and lambs. In conclusion, whilst regulation of sITLNs and sSIAT4C transcript expression in the mucosa may differ, these molecules may have an important role to play in the mucosal immune response to parasitic infections in sheep.

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DEDICATION

'Real knowledge is to know the extent of one's ignorance'

Confucius

To my children Robin and Alex Honhold and to the memory of my parents John and Una French.

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ABBREVIATIONS

AA	amino acid
AAM	altenatively activated macrophages
AB/PAS	alcian blue/periodic acid Schiff
AGP	alpha -1 acid glycoprotein
AMV	avian myeloblastosis virus
BAL	broncho-alveolar lavage
bp	base pair
cDNA	copy deoxyribonucleic acid
cnv	challenged naïve
cpi	challenged previously infected
Cys	cysteine
DAB	diaminobenzide
dNTP	dinucleotriphosphate
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EST	expressed sequence tag
FCS	foetal calf serum
FEC	faecal egg count
FIZZ	found in inflammatory zone
FM	free mucus
FW	forward
GC	goblet cells
GSP	gene specific primer
h	hour
HCl	hydrochloric acid
IFN	interferon
Ig	immunoglobulin

IL-	interleukin
ITLN	intelectin
kDa	kilo Dalton
KO	knock out
L3	stage 3 larva
mAb	monoclonal antibody
MAL	Maackia amurensis
MBL	mannose binding lectin
MCP	mast cell protease
min	minute
MRI	Moredun Reseach Institute
mRNA	messenger ribonucleic acid
MW	molecular weight
NaCl	sodium chloride
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycoylneuraminic acid
NFAT	nuclear factor of activated T cells
OD	optical density
OvGal	ovine galectin
PBS	phosphate buffered saline
PGE2	prostaglandin E2
qPCR	Quantitative PCR
QTL	quantitative trait loci
RANTES	regulated on activation of normal T cells expressed and secreted
RELM	Resistin like molecule
RIN	RNA integrity number
RT-PCR	reverse transcriptase polymerase chain reaction
RV	reverse
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SEM	standard error of the mean
SIAT4C	beta galactoside alpha 2-3 sialyltransferase

sLe	sialyl Lewis
SMG	sub mucosal glands
STAT	signal transducers and activators of transcription
TGF	transforming growth factor
Th	T cell helper
TI	trickle infected
TNF	tumour necrosis factor
unv	unchallenged naïve
UoE	University of Edinburgh
upi	unchallenged previously infected
vWF	Von Willebrand factor

1 REVIEW OF THE LITERATURE

1.1 Introduction

The mucosal surfaces have many roles including absorption, macromolecule transport, barrier and secretory functions. Physical and chemical barriers as well as innate and acquired immune systems control the response to the numerous allergens and pathogens to which they are exposed. Knowledge of the molecules and pathways involved in the mucosal immune response is important for our understanding of host defence against helminth infections as well as the mechanisms of debilitating diseases such as asthma, allergy and inflammatory bowel disease. Whilst recent research has increased our understanding of the role of pathogen–associated molecular patterns, Toll like receptors (de Veer et al., 2007), epithelial cells (Bals and Hiemstra, 2004), tight junctions, mucus (Theodoropoulos et al., 2001; Rogers, 2007), innate effector cells, antigen-presenting cells (macrophages and dendritic cells), (Maizels et al., 2004), T lymphocytes subsets and their cytokines (Neurath et al., 2002) and the interrelationship of all of these in the mucosal immune response, there are still many unknowns.

The gastrointestinal and respiratory mucosal response to allergens and parasites share similarities and are characterised by an increase in eosinophils, mast cells, goblet cells, mucus production and IgE (Else and Finkelman, 1998; Walter and Holtzman, 2005). The allergic response is thought to be due to a defect in the immunological balance between Th1 and Th2 lymphocyte subsets and the Th2 cytokines have been shown to be the key mediators of the response (Neurath et al., 2002). The role of the goblet cell in this response has been of particular interest from two very different perspectives; the formation of fatal asthma plugs in humans (Groneberg et al., 2004) and in the trapping and elimination of parasites in the gastro-intestinal tract (Miller et

al., 1981; Miller et al., 1983). Recent research has shown upregulation of intelectin (ITLN), RELM beta (RELMβ) and beta galactoside alpha 2-3 sialyltransferase (SIAT4C), all genes that have been associated with mucus producing cells, in gastro-intestinal parasitic infections in mice (Knight et al., 2004; Pemberton et al., 2004a). The hypothesis for this thesis is that these genes are co-regulated in the mucosa during a typical Th2 response.

In this literature review emphasis has been placed on mucosal immunity in general and specifically against parasites, as much of our understanding of mucosal immunity comes from parasite models. In addition to reviewing the literature pertaining to the molecules of interest, literature to their entire gene families has been reviewed.

1.2 Mucosal immunity against gastrointestinal parasites

1.2.1 Introduction

Gastrointestinal nematode parasites are important causes of disease in humans and domestic species (de Veer et al., 2007). In man, species belonging to the genera *Ascaris*, *Ancylostoma, Necator*, *Strongyloides* and *Trichuris* infect millions of people especially in developing countries causing growth retardation, impaired cognitive function, reduced productivity, immune hyporesponsiveness and death (Cooper and Bundy, 1988; Guyatt, 2000; McDonald, 2003).

Parasitic gastroenteritis is a major problem in farm animals worldwide causing significant production loss and the species *Haemonchus*, *Ostertagia*, *Teladorsagia* and *Trichostrongylus* are the main ones implicated in economic loss primarily due to the length of time taken to develop immunity (Gross et al., 1999; Claerebout and Vercruysse, 2000; Newton and Meeusen, 2003).

Control of gastrointestinal helminths in farm animals relies heavily on the use of anthelmintics, however increasing concerns regarding drug residues and emerging anthelmintic resistance (Wolstenholme et al., 2004; Coles, 2005; Wrigley et al., 2006) have encouraged investigation of alternative control strategies including vaccination (Knox and Redmond, 2006; Smith and Zarlenga, 2006; Vercruysse et al., 2007), natural anthelmintics (Shaik et al., 2006; Athanasiadou et al., 2007), nematode destroying fungi (Mendoza-De Gives et al., 2006; Stear et al., 2007b) and selective breeding for resistance (Bishop and Stear, 2003); Davies et al., 2006; Stear et al., 2007b).

Whilst the presence of gastrointestinal parasites in people are generally considered detrimental to health there is concern that elimination of parasites in the First world has led to dysregulation of the T cell response with a resulting increase in inflammatory bowel disease and auto-immune disease (Elliott et al., 2007).

Our understanding of the immune response to gastro-intestinal helminth infections is vital to development of new control strategies in ruminants and also in understanding the role of helminth infections in people. These are active areas of research using mainly rodent models of *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Heligmosomoides bakeri* and *Trichuris muris* infection.

1.2.2 Manifestations of mucosal immunity against gastrointestinal parasites

Typical manifestations of mucosal immunity include expulsion of adult parasites, stunting of parasites, decrease in female worm fecundity, failure of infective larvae to establish and arrested development of larvae. These mechanisms have been described in rodents, livestock and man. Whilst rapid expulsion of adult worms occurs in most rodent models, studies have shown that effector mechanisms may differ depending on the parasite (Else and Finkelman, 1998) and in the case of *T. muris* on the strain of mouse (Else et al., 1989). Interestingly in man this phenomenon of rapid expulsion is uncommon and chronic infections predominate

(Behnke et al., 1992), whilst in ruminants host genetics and species of parasite determine time to development of immunity.

Stunting of adult worms has been demonstrated in several livestock parasites including *Haemonchus contortus*, *Ostertagia ostertagi* and *Teladorsagia* (Stear et al., 1995; Gasbarre et al., 2001) and also in rodent models infected with *T. spiralis* in which a dose related response has been reported. A decrease in fecundity of female worms has been implicated as a major regulatory force for gastrointestinal nematode populations in sheep (Stear et al., 1995a) and has also been shown to occur in rodent models (Sato and Toma, 1990). Finally a major manifestation of acquired immunity is failure of infective larvae to establish and mature to adults in the gut. This is typically most strongly expressed in challenge infections but also occurs in primary infections. Interestingly in ruminants but not in rodents (Behnke and Parish, 1979) arrested larval development is common and is associated with increased resistance (Onah and Nawa, 2000).

1.2.3 Effector mechanisms in the gastrointestinal mucosa against parasitic nematodes

1.2.3.1 T cells, T helper subsets and the role of cytokines

Studies in rodent models have demonstrated that protective immunity against gastrointestinal parasites is T cell dependent and that $CD4^+$ T cells play a central role (Else and Finkelman, 1998). $CD4^+$ T cells can be segregated into two distinct T helper subsets, Th1 and Th2, based on their cytokine profile (Mosmann et al., 1986). The most potent influence on differentiation into Th1 and Th2 cells appears to be the immediate cytokine environment at the time of antigen presentation with interleukin – 12 (IL-12) promoting the formation of Th1 cells whilst Th-2 cells develop in the presence of IL-4 (Else and Finkelman, 1998). Studies of *T. muris* infection in different mouse strains have shown immune polarization of the mesenteric lymph node cell response towards Th2 in resistant and Th1 in susceptible mice (Grencis, 2001). Extensive studies in knock out and transgenic mouse have demonstrated that IL-4, IL-5, IL-9 and IL-13 are the Th2 cytokines that are of most important in a

resistant profile (Onah and Nawa, 2000) and in particular IL-4 and IL-13 which both bind to the IL-4Rα and thus activate the key cytoplasmic signal transducer and activator of transcription molecule 6 (STAT6) through phosphorylation by Janus kinases 1 and 3 (Khan and Collins, 2004). STAT6 translocates to the nucleus, where it binds to regulatory sites, in several different genes. The absence of IL-13, IL-4Rα or STAT-6 all disable protective immunity and have been shown to permit survival of *N. brasiliensis* in mice (Urban et al., 1998). Interestingly the administration of recombinant TNFα has been shown to enhance the IL-13 mediated protection against *T. muris* infection (Artis et al., 1999; Hayes et al., 2007).

Similar tendencies towards Th2 cytokine responses have been observed during parasitic infections in cattle (Almeria et al., 1997; Gasbarre et al., 2001; Claerebout et al., 2005) and in sheep (Gill et al., 2000; Pernthaner et al., 2005; Craig et al., 2007). In primary *Ostertagia* infection in cattle high levels of expression of IL-4 were found in both the draining lymph node and in lymphocytes isolated from the abomasal mucosa, however unlike the murine models, interferon-gamma (INFγ) was also found to be upregulated (Almeria et al., 1997; Canals et al., 1997).

In *Trichostrongylus colubriformis* infection of naive or primed sheep IL-5, IL-13 and TNFα were expressed and furthermore were consistently found at higher levels in genetically resistant compared to susceptible sheep (Pernthaner et al., 2005). Interestingly the genetically resistant sheep in their experiment were able to respond to experimental infection despite the presence of moderate levels of INFγ. Furthermore, nematode challenge resulted in a decrease in levels of IL-10, a cytokine which is typically associated with development of a polarized Th2 response and resistance in murine models (Schopf et al., 2002). Interestingly recent work in rodent experiments has highlighted the role of TNFα in augmentation of immune responses (Hayes et al., 2007).

Very different patterns of cytokine expression have been noted in sheep primed with *H. contortus* that rejected challenge larvae before they reached their tissue niche compared to sheep where tissue larvae were present (Balic et al., 2002). Sheep that

rejected larvae had higher levels of IL-4 at baseline and did not show significant upregulation of any cytokines, whilst sheep with larvae present showed upregulation of IL-4, IL-5, IL-13 and INFγ compared to baseline.

It is uncertain which cells commit the immune system to a final Th2 pathway in the presence of parasites. Dendritic cells, eosinophils, basophils, B cells, natural killer cells and mast cells have all been shown to be capable of producing IL-4 and have been implicated in amplification of the Th2 response and as effector cells in initiating the response (Bradding et al., 1992; Harris et al., 2000; Balmer and Devaney, 2002; Shinkai et al., 2002). A recent study in an *N. brasiliensis* murine model has shown that loss of expression of MHC class 11 rendered mice unresponsive to *N. brasiliensis* excretory secretory antigens whilst MHC class 1 knock out, B cell deficient and IL-5 deficient mice still showed a strong Th2 response (Holland et al., 2005).

An area of considerable interest is the role of regulator T cells (CD 25^{+}) in chronic parasitic infections. Through production of IL-10 and transforming growth factor-beta (TGFβ) these cells can switch off inflammatory and protective immune responses, and nullifying their activity can 'cure' parasite infection by permitting the immune system to act at full potential (Maizels et al., 2004).

1.2.3.2 Antibody responses

Intestinal nematode infections in murine models are accompanied typically by elevations in IgG1 and IgE, with both these isotypes under the control of Th2 cytokines, however there are few studies to indicate that antibodies represent a principal effector mechanism in resistance (Else and Finkelman, 1998). In fact studies have shown that IL-9 transgenic mice are able to mediate expulsion *of T. muris* in the absence of any detectable antibody response and transfer of immune CD4+ T cells to SCID mice protects them from a primary infection (Else and Finkelman, 1998). The binding of IgE to the high affinity IgE receptors (FcεRI) results in activation of mast cells, however mast cell activation has also been shown

to occur independently of IgE and it has been suggested that as most of the IgE produced is non specific and that it may actually block receptors, preventing specific IgE binding (Pritchard, 1993). Very little attention has been paid to local IgA antibody production in rodent models, however it is interesting that in a non responsive mouse strain, passive transfer of immunity to *T. muris* could be achieved with IgA monoclonal antibodies (mAb) specific to E/S antigens where IgG and IgM mAbs were unsuccessful (Roach et al., 1991).

Ruminants have also been shown to develop local and general specific antibody responses. Again their relative importance in host protection is still unclear. In an experimental *T. circumcincta* infection in sheep the number of inhibited larvae was shown to be positively associated with the size of the local IgA response (Stear et al., 2004). *In vitro* studies in abomasal lymph cells from lambs genetically resistant to *H. contortus* have shown production of significantly more IgG and IgE than similar cells from random bred animals at day 28 post infection (Gill et al., 2000). Whilst higher levels of circulating IgE bearing cells have been observed in responder lambs (lambs that develop a response more rapidly and express a more effective immunity against gastrointestinal parasites) compared to non-responders, numbers failed to reach significance (Pettit et al., 2005).

1.2.3.3 Eosinophils

A local and circulatory eosinophilia and also upregulation of IL-5 the cytokine responsible for generation of eosinophils has been demonstrated in murine and livestock nematode infections (Stear et al., 1995; Meeusen and Balic, 2000; Scott et al., 2000; Sugaya et al., 2002). Extensive studies in rodent models using IL-5 KO mice, IL-5Rα KO mice, IL-5 transgenics and mice treated with anti-IL-5 mAb have shown that eosinophils do not appear to play a role in worm expulsion (Else and Finkelman, 1998; Onah and Nawa, 2000) however there is some evidence to suggest that they may mediate a protective immune response against migratory larval stages of some nematodes (Meeusen and Balic, 2000; Sugaya et al., 2002). A recent study has shown that resistance to reinfection with *Necator americanus* in people is associated with a parasite specific IL-5 response (Quinnell et al., 2004). It is of

interest that adult stages of *T. circumcincta* and excretory secretory material from *T. circumcincta* L3 have been shown to produce potent chemoattractant activity for bone marrow derived eosinophils *in vitro* raising the question of whether eosinophils may be permissive rather than protective against the parasites (Wildblood et al., 2005).

1.2.3.4 Mast cells

Increased numbers of mucosal mast cells are observed during gut nematode infections and controlled by a variety of Th2 cyokines including IL-3, IL-4, IL-9, IL-10 and the growth factor, stem cell factor (SCF) (Else and Finkelman, 1998). The role of mast cells in expulsion of parasites differs between parasite species and this has been shown clearly in several studies using STAT6 KO, *c-kit* KO and mice treated with anti-IL-3 mAb or anti-*c-kit* antibodies (Else and Finkelman, 1998). The activation of mast cells may be mediated by IgE or by IgE independent mechanisms and activated mast cells are thought to play a role in resistance to infection through the release of inflammatory mediators which increase permeability of the gut epithelium (Scudamore et al., 1995). It has also been hypothesised that the highly sulphated proteoglycans released from mast cell granules may be effector molecules that prevent the establishment of *Strongyloides* in mice and rats (Maruyama et al., 2000; Onah and Nawa, 2000) as heavily sulphated goblet cell mucins have been shown to result in more rapid expulsion of *Strongyloides* in rodent models (Ishikawa et al., 1994). Mast cells can also secrete cytokines such as IL-4 and IL-5 and interestingly the muscle larvae specific antigens of *T. spiralis* have been shown to induce IL-4 and TNF in murine mast cell lines (Niborski et al., 2004).

Increased mucosal mast cell numbers are also recognised in ruminant gastrointestinal parasitic infections. Studies in sheep have shown that challenge infection of sheep immune to *H. contortus* and *T. colubriformis* results in rapid expulsion of the majority of challenge larvae before they reach their tissue niche with limited lymphocyte recruitment, this response has been associated with the presence of intraepithelial mucosal mast cells (globule leukocytes) and has been suggested to be an immediate type 1 hypersensitive reaction (Huntley, 1992).

1.2.3.5 Goblet cells

Increased goblet cell numbers and mucin production are features of many gastrointestinal nematode infections (Else and Finkelman, 1998) however the role of the goblet cell in the protective immune response has not received much attention. Mucus trapping of parasites during the rapid expulsion of *N. brasiliensis* from primed rats has been demonstrated (Miller et al., 1981), whilst other authors have demonstrated an association between sulphation of the goblet cell mucins and worm expulsion (Koninkx et al., 1988; Ishikawa et al., 1994).

Studies in IL-4Rα, IL-9 and IL-13 deficient mice infected with *N. brasiliensis* have shown the importance of the Th2 cytokines in increasing goblet cell numbers and furthermore experiments with *T. spiralis* infected, STAT6 deficient mice, have shown that this is mediated through a STAT6 pathway (Townsend et al., 2000; Khan et al., 2001). Interestingly IL-4 KO mice infected with *T. spiralis* showed an increase in goblet cell numbers comparable to wildtype (Khan et al., 2001). Of particular interest is the ability of athymic rnu/rnu rats infected with *N. brasiliensis* to develop marked goblet cell hyperplasia which is not protective (Kawai et al., 2007).

There are at least twelve genes encoding mucin proteins in man that have been recognised to date (Corfield et al., 2001). Two distinct genes exist in the intestinal tract, named MUC2 and MUC3 in humans and Muc2 and Muc3 in mice. Muc2 is a secretory mucin and Muc3 is membrane bound and both have been shown to be upregulated in *T. spiralis* infection in either wildtype or IL-4 KO mice (Shekels et al., 2001). Muc2 and Muc3 are also upregulated in rats infected with *N. brasiliensis* and furthermore upregulation was seen in euthymic and athymic rats suggestive that intestinal mucin production can occur without the activation of thymus derived T cells (Kawai et al., 2007).

Recent microarray studies of the murine intestinal epithelium in nematode infections have shown upregulation of several gobet cell related genes such as Muc3, Gob4, Gob5, calcium chloride channelA3, SIAT4C, ITLN1b/2 and RELMβ (Knight et al.,

2004; Datta et al., 2005; Artis, 2006; Yamauchi et al., 2006; Kawai et al., 2007). It has been hypothesised that the marked early upregulation of ITLN1b/2, in *T. spiralis* infected mice may be associated with alteration in the character of the mucus leading to entrapment of parasites (Pemberton et al., 2004a) whilst *in vitro* studies have suggested that RELMβ may play a role in altering the chemosensory function of parasites (Artis et al., 2004).

1.2.3.6 Macrophages

Macrophages may be activated by pro-inflammatory signals such as TLR ligands and IFNγ, 'classical activation', however they may also be activated by Th2 cytokines. The phenotype of the macrophages seen in a Th2 response differs, they are termed alternatively activated macrophages (AAMs) and are characterised by their ability to upregulate arginase (Nair et al., 2006). Suggested roles in parasitic infection have included suppression of inflammation, effector cells against parasites, amplification of a Th2 response and tissue repair (Maizels et al., 2004; Nair et al., 2006).

1.2.3.7 Gut secretions

Increase in gut water and electrolyte secretions is a common feature of nematode infections, responses occurring within days in primary infections and within minutes in secondary infections in rodent models (Castro et al., 1979). Studies have shown that mast cell amines are Cl^- secretagogues and work through enteric nerves to stimulate Cl^- secretion within minutes after antigen challenge in the gut, which is followed by a second phase of secretion mediated by PGE2 (Castro et al., 1987). Chloride ion secretion results in secretory diarrhoea which may flush the intestine of parasites or produce a hostile environment (Baird and O'Malley, 1993).

1.2.3.8 Intestinal motor system

There is evidence from experimental studies for the involvement of the intestinal motor system in defence against nematode infections and that this is partly under intrinsic nerve control (Alizadeh et al., 1987). In *T. spiralis* infected mice intestinal

muscle hypercontractility was noted in the proximal small intestine and muscle contractility was found to be greater in strong responder strains compared to weak responders. Furthermore, studies using athymic and $CD4^+$ deficient mice showed that $CD4^+$ T cells play a role in enteric muscle function (Vallance and Collins, 1998). There is now evidence to suggest that IL-4 plays a direct role in smooth muscle hyper-responsiveness in the small intestine (Vallance et al., 2007).

1.3 Mucosal immunity in allergic disease of the respiratory tract in man

Asthma was originally considered to be a muscular or a neurologic disorder and was subsequently characterised as an inflammatory condition that may be allergic or non-allergic in origin (Walter and Holtzman, 2005). Allergic asthma is characterised by airway inflammation, increased goblet cell numbers, increase mucus production, airway hyperreactivity (AHR) and bronchial constriction. There is an early acute response with mast cells and IgE implicated and a late response involving eosinophils, Th2 lymphocytes and their cytokines IL-4, IL-5, IL-9 and IL-13 (Walter and Holtzman, 2005). Various transcription factors such as GATA-3 and STAT6 are known to induce or augment Th2 cytokine production and studies have shown upregulation of these factors in patients with asthma (Neurath et al., 2002). In mouse asthma models STAT6 deficient mice are protected from Th2 mediated bronchial inflammation and airway hyperreactivity and targeting of GATA-3 expression in the lung using antisense oligoneucleotides has led to suppression of established airway inflammation, AHR and IL-4 production (Neurath et al., 2002).

Animal models of asthma have identified numerous other possible targets for therapeutic intervention including cytokine and goblet cell inhibitors. Different species of animals have been used as models in asthma research and the sheep has been shown to be a particularly good model (Bischof et al., 2003; Rosen et al., 2005). Several researchers have focused on the goblet cell because of the association between increased mucus production and fatal asthma plugs (Rose et al., 2001; Rogers, 2007). Whilst asthma was originally thought of as of an entirely reversible

disorder, several longitudinal studies have shown an accelerated rate of respiratory functional deterioration in asthmatics associated with chronic airway remodelling. This is an active area of research and a better understanding of the pathophysiology of this remodelling will allow identification of appropriate therapeutics options to target the chronic changes associated with this disease (Elias et al., 1999).

The genetic component of asthma has received considerable interest and there is unequivocal evidence from population studies that asthma has a genetic component and to date at least 64 human genes have been identified that associate with asthma (Walter and Holtzman, 2005). Of particular interest is a recent human study which showed high heritability of the Th1 cytokines TNFα, IFNγ and T bet, the transcription factor which controls IFNγ production in CD4 T cells. In contrast little or no influence of genetic factor was found on transcription factors known to be involved in regulation of Th2 cytokines such as nuclear factor Kappa B, nuclear factor of activated T-cells (NFAT) and GATA3 suggesting that genetic control of human Th cell development may reside mainly in the regulation of Th1 cytokines (Hohler et al., 2005).

1.4 The hygiene hypothesis

The concept that higher standards of personal cleanliness with decreased cross infection in young families was associated with a higher prevalence of atopy was first published in 1989 and was the introduction of the 'hygiene hypothesis' (Strachan, 1989). Since that time there has been considerable interest in the relationship between the eradication of parasitic disease, polio, malaria and tuberculosis in the Western world and the increase in prevalence of asthma, inflammatory bowel disease, type 1 diabetes and multiple sclerosis (Palmas et al., 2003). Investigative studies have shown that helminths can down regulate intestinal and respiratory tract inflammation and prevent development of several immune mediated diseases due to a shift of Th cell profile with an increase in Th2 and T reg cells (Wilson et al., 2005; Elliott et al., 2007; Setiawan et al., 2007) and this has led to investigation of new treatment modalities and to clinical trials using helminths to

treat inflammatory bowel disease (Shea-Donohue and Urban, 2004; Summers et al., 2005a; Summers et al., 2005b).

1.5 Intelectin

1.5.1 Introduction

Upregulation of mouse ITLN1b/2 gene transcript was shown in the jejunum of mice infected with *T. spiralis* infection, as early as day three post infection, and was immunolocalised to mucus producing cells (Pemberton et al., 2004a; Pemberton et al., 2004b). The reason for this upregulation is unknown. The following is a review of the different members of the ITLN family and current knowledge regarding expression and putative function.

1.5.2 Lectins – an overview

Lectins have important diverse functions including cell-cell adhesion and pathogen surveillance (Varki et al., 1999). Several families are recognised based on their amino acid homology and probable evolutionary relatedness; C-type, S-type, P-type, I-type, hyaluronon binding, calnexin and frog egg lectin. The C type lectins such as collectins and ficolins are soluble and function as opsonins or agglutinins for bacteria. The mannose binding lectin (MBL), a typical collectin, forms complexes with MBL associated serine proteases in plasma. The binding of these complexes to targets activates the complement system and induces opsonization of targets by phagocytes. This pathway is known as the lectin pathway (Varki et al., 1999). On the other hand selectins, another C-type lectin are extensively involved in cell trafficking. The galectins (galactose binding lectins) have been shown to have very diverse functions participating in cell growth regulation, apoptosis (Perillo et al., 1998) and binding to gastrointestinal tract mucins (Wasano and Hirakawa, 1997). The ITLN family are composed of calcium dependent galactose binding lectins and the functions of many members of this family still remain to be determined.

1.5.3 The ITLN family

ITLNs appear to have arisen early in chordate evolution, homologues are known in sea squirts, fishes (Chang and Nie, 2007; Gerwick et al., 2007), frogs (Chang et al., 2004) and mammals (Komiya et al., 1998; Lee et al., 2001; Suzuki et al., 2001). Whilst in most species only one homologue has been shown, two homologues have been described in man; human ITLNs 1 (hITLN1) and 2 (hITLN2) (Lee et al., 2001; Suzuki et al., 2001; Tsuji et al., 2001) and in mice; mouse ITLNs 1a (mITLN1a) and 1b/2 (mITLN1b/mITLN2) (Komiya et al., 1998; Pemberton et al., 2004a). hITLN1 has also been named human small intestinal lactoferrin receptor (Suzuki et al., 2001), HL1 of vascular endothelial cells (Lee et al., 2001) and omentin of adipose tissue (Schaffler et al., 2005) whilst mITLN1 is also known as mouse lactoferrin receptor (Suzuki and Lonnerdal, 2004).

Whilst ITLNs are calcium dependent galactose binding lectin, it is not a member of the C-type lectin or galectin families. hITLN1 has been shown to bind galactofuranosyl residues, which are not found in mammalian tissues, as well as D-xylose, and both D- and L-ribose, but mITLN appears to have different carbohydrate binding specificities (Tsuji et al., 2007). Interestingly, whilst most of the ITLN homologues exist as oligomers (Chamow and Hedrick, 1986; Abe et al., 1999; Suzuki et al., 2001; Tsuji et al., 2001; Nagata, 2005), mITLN1a has been shown to exist as a 34kDa non-glycosylated monomer. It is unknown if these differences alter function (Tsuji et al., 2007).

1.5.4 Tissue distribution of the ITLNs

mITLN1a when first reported was found to be constitutively expressed in intestinal Paneth cells (Komiya et al., 1998). More recently transcript expression has been shown in the intestine, lung, spleen, kidney, heart and testis (Suzuki and Lonnerdal, 2004). On the other hand, in the normal animal mITLN1b/2 transcript has only been shown in the respiratory tract (Pemberton et al., 2004a; Voehringer et al., 2007). In mice expression has been immunolocalised to small intestine, kidney, stomach,

salivary glands, pancreas, oesophagus, bladder, colon, ovaries, prostate, brain, spleen and lymph node (Suzuki and Lonnerdal, 2004).

Studies have shown a wide constitutive expression of hITNL1 transcript, including heart, colon, small intestine, thymus, ovary, testis, spleen, lymph node and stomach whilst hITLN2 has been detected exclusively in the small intestine (Lee et al., 2001). In man protein expression was initially reported restricted to endothelial tissue (Lee et al., 2001), however expression has more recently been shown in respiratory epithelial cells, mesothelial cells, omental fat and serum (Kuperman et al., 2005; Wali et al., 2005; Yang et al., 2006).

ITLN is also expressed in pigs and has been immunolocalised to intestinal paneth cells, goblet cells and as a novel lipid raft associated protein in the enterocyte brush border. In the same study ITLN was also immunolocalised to the proximal tubule cells of the kidney (Wrackmeyer et al., 2006). Interestingly in the grass carp, *Ctenopharyngodon idella,* ITLN transcript and protein has also been shown in the head kidney and trunk kidney as well as gill, intestine, brain, spleen and heart (Chang and Nie, 2007).

1.5.5 Function of ITLNs

1.5.5.1 ITLN and the reproductive tract in Xenopus laevis

The *Xenopus* lectin is thought to play a role in defence against polyspermy by alteration of the character of the jelly coat layer surrounding the oocyte (Nishihara et al., 1986).

1.5.5.2 ITLN and innate immunity against microorganisms

Recombinant hITLN1 had been shown to recognise D-galactofuranosyl residues present in the arabinogalactan on the cell wall of *Nocardia rubra* (Tsuji et al., 2001). Interestingly galactofuranose residues are present within the carbohydrate chains of a variety of microorganisms such as *Mycobacteria* (Daffe et al., 1993), *Streptococcus*

(Abeygunawardana et al., 1991)), *Leishmania* (Suzuki et al., 2002) and *Trypanosoma* (de Lederkremer and Colli, 1995) however are lacking in mammals. Studies in *Leishmania major* have shown that when the gene LPG1, which encodes a galactofuranosyl transferase involved in the formation of the GPI anchored cell surface polysaccharide, lipophosphoglycan, was removed by targeted gene disruption, there was loss of virulence (Spath et al., 2000). These findings may support a role for ITLN in innate defence.

It is interesting that the ascidian plasma lectin has been shown to increase phagocytic activity of *Halocynthia roretzi* hemocytes towards sheep red blood cells (Abe et al., 1999).

1.5.5.3 ITLN as a lactoferrin receptor

ITLN has been identified as a lactoferrin receptor in man, mouse and in pigs (Suzuki et al., 2001; Suzuki and Lonnerdal, 2004; Liao et al., 2007). Lactoferrin is a member of the transferrin family. It was originally thought to facilitate iron absorption in infancy and as an iron binding protein to have bacteriostatic effects, however there is increasing evidence for several physiological roles. It has been shown to modulate iron homeostasis, to have anti-microbial activities, anti-inflammatory activity, to stimulate cytokine production, regulate cell growth and protect against cancer (Ward et al., 2005). The biological activity of lactoferrin may depend on the target cell and the binding to the specific lactoferrin receptors. It is unknown what role ITLN, as a lactoferrin receptor, plays in the proposed functions of lactoferrin.

1.5.5.4 ITLN in the acute phase response in fish

The acute phase response is conserved through vertebrate evolution and enhances the ability to fight infectious agents and to heal wounds (Jensen et al., 1997). In a recent microarray study in rainbow trout, ITLN was found to one of the acute phase proteins upregulated in the liver 24 hours after intraperitoneal injection with *Listorella anguillarum* (Gerwick et al., 2007). It was postulated that ITLN may have been involved in the sequestration of iron in its role as a lactoferrin receptor.

1.5.5.5 ITLN and gastro-intestinal parasite infections in mice

In a proteomic analysis of small intestinal epithelium, upregulation of mITLN1b/2 in BALB/c mice was shown at day 14/15 post *T. spiralis* infection (Pemberton et al., 2004b) A further study showed upregulation of gene transcript in the jejunum as early as day three post infection suggestive of an innate response (Pemberton et al., 2004a). The BALB/c mice are considered a resistant strain resulting in early expulsion of parasites. Interestingly, mITLN1b/2 was not detected in the slower responding C57BL/10 strain following *T. spiralis* infection and genomic studies showed that mITLN1b/2 was absent from the genome of this strain (Pemberton et al., 2004a). `

Two independent recent micro-array analyses, of intestinal responses following *T. muris* infection in mice, have shown marked upregulation of ITLN in response to infection (Datta et al., 2005; Artis, 2006). Interestingly differential responses were seen between mouse strains with upregulation of ITLN in the resistant BALB/c mouse strain but not in the susceptible AKR strain (Datta et al., 2005). These findings are supportive of a protective role for ITLN in the innate immune response to parasite infections.

Infection with *Nippostrongylus brasiliensis* also results in upregulation of mITLN1b/2 transcript in the small intestine and upregulation of both mITLN1a and mITLN1b/2 in the lung and furthermore comparative studies using wild type and STAT6-deficient mice have confirmed that upregulation is STAT6 dependent (Voehringer et al., 2007). Interestingly overexpresson of either mouse ITLN in the lung did not alter the kinetics of worm expulsion (Voehringer et al., 2007). Further studies might clarify if these findings apply to the intestinal tract and to other species.

1.5.5.6 ITLN and asthma in people

Increase in ITLN transcript expression both on microarray and on RT-PCR has been shown in a wildtype mouse ovalbumin asthma model (Kuperman et al., 2005). When

wildtype were compared to IL-13 transgenics, there was a greater increase in expression in transgenic IL-13 mice following allergen challenge and the greatest increase was seen in transgenic IL-13 mice, with STAT 6 expression restricted to the respiratory epithelium, confirming the regulation of ITLN by IL-13 and the epithelial localisation of ITLN.

To see if there was similar expression of ITLN in humans, the same authors cultured human airway epithelial cells with the Th2 cytokine, IL-13 and found upregulation of hITLN. They also found upregulation of hITLN gene expression by real time PCR in the bronchial brushings from 30 asthmatic people compared to 28 controls (Kuperman et al., 2005).

In a proteomic analysis of the supernatant of bronchoalveolar lavage fluid samples of asthmatic patients, hITLN1 was found to be present at baseline and upregulated following allergen challenge, a finding that confirms the secretion of ITLN into the lumen of the airways (Wu et al., 2005).

The upregulation of hITLN in asthma, a known Th2 response, suggests that the mouse intestinal tract ITLN response is unlikely to be parasite specific, but part of a general Th2 response. It is unknown if the upregulation of hITLN in asthma may result in alteration of mucus character and thus play a role in the formation of fatal asthma plugs.

1.5.5.7 ITLNs and neoplasia in people

Recent work has shown weak expression of hITLN in normal mesothelium and overexpression in malignant pleural mesothelioma. hITLN was immunolocalised within the cytoplasm and/or at the membrane borders of cells (Wali et al., 2005). There is a well established association between mesothelioma and exposure to asbestos and interestingly there was upregulation of hITLN1 when crocidolite asbestos was added to primary mesothelial cell cultures. On examination of more tissues by multiple tissue micro-array, the authors found several normal gastro-intestinal tissue samples and cancerous tissue samples positive for hITLN. At the

moment it is unknown what role hITLN may play in the pathogenesis of neoplasia or whether it may have a potential prognostic or therapeutic application in selected cases.

1.5.5.8 ITLN and senescence

In a mouse model of senescence, a micro-array analysis of intestinal epithelium, confirmed by quantitative PCR, demonstrated significant down regulation of mITLN in the colonic epithelium of the senescent accelerated model SAM P6 mice compared to wild type controls (Kawashima et al., 2004). The significance of this finding is uncertain.

1.5.5.9 ITLN in obesity and diabetes mellitus

There has been a marked increase in interest in the biology of adipose tissue since the discovery in 1994 of the adipokine leptin. Many more adipokines have now been discovered including adiponectin, resistin, visfatin, apelin and omentin (ITLN) (Gualillo et al., 2007). Adipokines are thought to play a role in insulin resistance and the prevalence of type II diabetes mellitus (Wurm et al., 2007). Omentin transcript and protein have been identified in omental fat tissue and recombinant omentin has been shown to augment insulin mediated glucose uptake *in vivo* using adipocyte cultures (Schaffler et al., 2005; Yang et al., 2006). The presence of omentin in the plasma has been demonstrated by Western blot and ELISA and high constitutive levels were shown in insulin sensitive slim young adults (Wurm et al., 2007) whilst plasma levels are decreased with obesity (de Souza Batista et al., 2007) . Plasma levels have been shown to correlate positively with adiponectin and negatively with body mass index, waist circumference and insulin resistance (de Souza Batista et al., 2007). Future studies will hopefully clarify if omentin has a protective role against diseases such as type II diabetes mellitus.

1.6 Beta-galactoside alpha 2-3 sialyltransferase

1.6.1 Introduction

Beta-galactoside alpha 2-3 sialyltransferase (SIAT4C) has been shown to be markedly upregulated in the jejunal epithelium of mice infected with *T. spiralis*. This marked upregulation was seen as early at day 3 and persisted until expulsion of the parasite at Day 14 thereafter decreasing gradually to baseline levels (Knight et al., 2004). The very early induction of SIAT4C would suggest that it was related to the innate defence system. Previous studies have demonstrated that SIAT4C plays a role in the sialylation of mucins and thus it was hypothesised that upregulation of SIAT4C in *T. spiralis* infected mice was associated with sialylation of the goblet cell mucins. The following is a general literature review pertaining to reported changes in sialylation and expression of SIAT4C.

1.6.2 Sialyltransferases – an overview

The sialyltransferases are Golgi glycosyltransferases and twenty distinct sialyltransferases have been identified in both human and murine genomes (Harduin-Lepers et al., 2005). These enzymes catalyse transfer of sialic acid to the outermost end of N-glycans, O-glycans and glycosphingolipids (Varki et al., 1999). As there are more than forty known possible modifications of sialic acids, when uncertain of the type of sialic acid present at a location, then the generic abbreviation Sia is usually used, however for the purpose of this literature review the term sialic acid will be used. Specific sialyltransferases catalyse the attachment of sialic acid from the 2-carbon to underlying sugar chains in different alpha linkages. The most common linkages are to the 3- or 6- position of galactose residues (Varki et al., 1999). Of particular interest in this study is the sialylation of mucins which are high molecular weight transmembrane or secreted proteins that are extensively O-glycosylated.

1.6.3 Sialic acid

Sialic acids are electronegatively charged monosaccharides present in higher animals and some microorganisms. The most commonly occurring members of the Sialic acid family are N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and N-acetyl-9-O-acetylneuraminic acid ($Neu5,9Ac_2$) (Schauer, 2004), however man has lost the expression of Neu5Gc due to a gene mutation (Irie et al., 1998). Interestingly the cow is the animal with the highest number of different sialic acids known to date with 15 types detected in the submandibular gland mucins (Klein et al., 1997).

The external position of sialic acid allows interaction with components of other cell surfaces, extracellular substances and effector molecules. Due to the negative charge sialic acid is involved in the binding and transport of positively charged molecules, such as pharmaceuticals, as well as in the attraction and repulsion of cells and molecules (Kelm and Schauer, 1997). Sialic acid contributes to the high viscosity of mucins lining and protecting endothelium (Schauer, 2004).

Sialic acid also acts to mask recognition sites which may be of both physiological and pathological importance, for example desialylation of red blood cells results in uptake by phagocytes and rapid clearance from the circulation (Bratosin et al., 1998) whilst desialylation by bacterial or viral sialidases may expose cellular antigens resulting in auto antibody formation. On the other hand, the over sialylation of malignant cells may protect them from humoral and cellular defence mechanisms. It is thought that reversible sialylation may regulate cell adherence and mobility during embryogenesis and malignant growth (Kelm and Schauer, 1997) and furthermore the type of sialic may differ in rapidly growing normal cells as well as in tumours (Kohla et al., 2002).

Studies in man have shown differential expression of sialic acid on mucins throughout the intestinal tract with an increasing expression of sialylated structures along the descending intestinal tract. Furthermore the rectal sialylated oligosaccharides were shown to be significantly more *O*-acetylated than in any other

part of the intestinal tract (Robbe et al., 2004). An increasing gradient of sialyl glycoconjugates and α2-3/α2-6 sialyltrasferase activity from the duodenum to colon has been shown in young suckling mice and interestingly a developmental decline in expression was shown to occur in the small intestine however the colon remained unchanged (Nanthakumar et al., 2003).

Sialic acid serves as a ligand for bacterial adhesions (Sakarya and Oncu, 2003) and for viral haemagglutinins such as the influenza virus (Suzuki et al., 2000). The numerous *N*- and *O*- glycans on mucins throughout the tracheobronchial and gastrointestinal mucosal epithelium provide ligands for a wide variety of microorganisms which can then be eliminated with the mucus (Lamblin et al., 2001). Airway mucins from cystic fibrosis or chronic bronchitis patients with severe bacterial infections have been shown to be highly sialylated (Davril et al., 1999). The altered sialylation could be of benefit to the invading organism, it may be a response from the host to circumvent the infection or might just be part of a general host response.

Transient alteration of sialylation has been reported in intestinal parasite infections (Karlsson et al., 2000). In a study of *N. brasiliensis* in rats, oligosaccharides were released from the intestinal insoluble mucins and characterised during the course of the infection. There was a gradual shift from NeuGc as the major sialic acid towards NeuAc during the infection, which normalised after the expulsion of the parasite. There was also a concurrent de novo expression of four sialylated oligosaccharides all containing the epitope NeuAcα2-3(GalNacβ1-4)Galβ1-, this epitope has previously been described in the Sd^{a-} and Cad-blood-group antigens. These changes were thought to be secondary to alterations in the expression of CMP-NeuAC hydroxylase and GalNac transferase respectively (Karlsson et al., 2000).

Sialic acid also functions as a ligand within animals, recognising siglecs and selectins. Many of the siglecs are involved in the recognition of various white blood cell types and macrophages (Crocker et al., 2007) whilst the selectins are involved in leukocyte–leukocyte interaction and leukocyte-endothelial interaction (Kelm and

Schauer, 1997). There is considerable interest in the role of selectin inhibitors to ameliorate the inflammatory response in diseases such as asthma (Abraham et al., 1999; Rosen et al., 2005) and also in the alteration of selectin ligands by gene therapy in order to decrease metastasis of tumour cells (Mathieu et al., 2004).

1.6.4 Sialic acid in an α2-3 linkage

Sialic acid is found in an α2-3 linkage in many if not all tissues in vertebrates (Varki et al., 1999). It has been implicated in the formation of the glycan ligands of the selectin family and in contributing to bacterial pathogenesis supporting adhesion of *Helicobacter pylori* and *Vibrio cholerae* toxin. Members of at least five different α2-3 sialyltransferases are responsible for synthesis of α2-3 linkages; ST3Gal-1, ST3Gal-11, ST3Gal-111, ST3Gal-IV and ST3Gal-V (Varki et al., 1999). Different nomenclatures are used for the sialyltransferases and using the HUGO gene nomenclature committee (HGNC) gene family nomenclature, the sialyltransferases responsible for α2-3 linkages in man are: SIAT4A (ST3Gal-1), SIAT4B (ST3Gal-11), SIAT4C (ST3Gal-IV), SIAT6 (ST3-Gal-III) and SIAT9 (ST3Gal-V). The sialyltransferases SIAT6 (ST3Gal-111) and SIAT4C (ST3Gal-IV) are expressed in most tissues and cells in adult mammals (Varki et al., 1999).

1.6.5 Beta-galactoside alpha 2-3 sialyltransferase – SIAT4C

1.6.5.1 SIAT4C transcript expression

Beta-galactoside alpha 2-3 sialyltransferase (SIAT 4C, ST3Gal-IV, CGS23, SIAT4, NANTA3, STZ, SAT3) has been shown to be expressed in a wide variety of human tissues (Kitagawa and Paulson, 1994a) and to have nine alternatively spliced transcripts with differential expression patterns (Grahn and Larson, 2001). The B transcripts have been found in all tissues examined whilst the A1 and A2 transcripts had more limited tissue distribution (Grahn and Larson, 2001). Interestingly in a micro-array study of glycosyl and sulfotransferases in normal human colonic tissue SIAT4C was found to be the most abundantly expressed enzyme (Kemmner et al., 2003). Murine SIAT4C gene expression has also been found to be widespread among

tissues and cell types with highest levels found in the small intestine and colon (Ellies et al., 2002).

1.6.5.2 SIAT4C and parasitic infections

Upregulation of SIAT4C has been shown in the jejunum of *T. spiralis* infected mice ((Knight et al., 2004) and *N. brasiliensis* infected rats (Yamauchi et al., 2006; Kawai et al., 2007). Upregulation occurred within 2-3 days after infection and reached highest levels at the time of parasite rejection. It is possible that the glycosylation status of the terminal sugar chains of mucins or membrane glycoproteins may have a role to play in rejection of parasites. Upregulation of SIAT4C was also shown in euthymic and athymic *N. brasiliensis* infected rats, suggestive that SIAT4C may be regulated by both thymus dependent and independent mechanisms (Kawai et al., 2007) and interestingly higher expression of SIAT4C transcript was seen in the euthymic rats.

1.6.5.3 SIAT4C and the Sialyl Lewis X (sLe^x) epitope

SIAT4C has been shown to be involved in the biosynthesis of the Sialyl Lewis X (sLe^X) epitope (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc) in man (Sasaki et al., 1993). sLe^X is a tetrasaccharide composed of galactose, *N*-acetylglucosamine, fucose, and sialic acid which is expressed on the surface of all circulating leukocytes and is also known to be elevated in tumour cells, particularly carcinomas. This epitope is expressed on normal colonic and respiratory tract epithelial mucins and is upregulated in patients with severe lung infections (Delmotte et al., 2001). Increased expression has also been shown on the serum acute phase protein alpha 1–acid glycoprotein (AGP) during inflammation (De Graaf et al., 1993). *In vitro* studies in rats have shown that IL-1 β and IL-6 increase expression of this epitope on AGP, however this was thought to be due to increased fucosylation rather than increased sialylation (Azuma et al., 2000). More recently IL-1β has been shown to upregulate SIAT4C gene transcription and to directly increase expression of the sLe^X complex in the HuH-7 hepatic carcinoma cell line (Higai et al., 2006). Interestingly SIAT4C transcript has also been shown to upregulate in human bronchial explants stimulated

with the proinflammatory cytokine, TNFα (Delmotte et al., 2002). There are no reports on the effects of other cytokines on expression of this molecule.

The sLe^x epitope expressed on circulating leukocytes has been demonstrated to serve as a ligand for selectins on endothelial cells and platelets (Foxall et al., 1992). In functional studies using a SIAT4C knock out mouse, SIAT4C deficiency resulted in reduction in the formation of selectin ligands on neutrophils and also resulted in an increase in E-selectin dependent leukocyte rolling velocity (Ellies et al., 2002b) The initial tethering of leukocytes to the endothelium was not affected. More recent studies have shown that SIAT4C is essential for interaction between L-selectin and the P-selectin glycoprotein ligand-1 (PSGP-1) which is present on adherent leucocytes and leucocyte fragments (Sperandio et al., 2006).

1.6.5.4 SIAT4C deficiency

Interestingly SIAT4C deficiency resulted in thrombocytopenia and decreased levels of Von Willebrand factor (vWF) which was shown to be secondary to exposure of galactose, which triggered asialoglycoprotein receptor (ASGPR) clearance mechanisms, thereby reducing levels of vWF and platelets in the circulation (Ellies et al., 2002a).

1.6.5.5 SIAT4C and lymphocytes

In vitro studies in murine splenic B lympocytes have shown upregulation of expression of SIAT4C following activation and significant correlation with the binding of *Maackia amurensis*, a lectin specific for sialic acid linked α2-3 to Galβ1-4GlcNAc, Galβ1-3GlcNAc and Galβ1-4GalNAc. Whilst there was lack of expression of SIAT4C in naïve murine T lymphocytes with rapid upregulation upon activation (Blander et al., 1999) They also showed rapid upregulation of α1-3 fucosyltransferase V11 (FucT-VII) the other enzyme involved in the formation of the sLe^x epitope. Furthermore they demonstrated that differentiation into Th1 and Th2 phenotypes resulted in differential expression of the two glycosyltransferases with SIAT4C expression remaining high in both phenotypes, whilst FucT-VII was

downregulated in Th2 cells, perhaps explaining the differential migration pattern of Th cells in tissues.

1.6.5.6 SIAT4C and neoplasia

There has been considerable interest in the expression of sialyltransferases as markers of malignancy in cancer cells. Studies in humans have shown that SIAT4C is downregulated in squamous cell carcinoma of the cervix (Wang et al., 2005), renal carcinoma (Saito et al., 2002) and colorectal cancer (Kemmner et al., 2003). Interestingly the sLe^x epitope has been shown to be upregulated in colonic carcinomas, however whilst SIAT4C has been shown to be involved in formation of this epitope on mucins in normal colonic epithelium, it did not appear to be involved in the increased expression of this epitope in the abnormal tissue (Kudo et al., 1998).

1.7 Resistin like molecule - beta

1.7.1 Introduction

Resistin like molecule – beta (RELMβ) gene expression can be induced by nematode parasites in murine models and protein expression has been immunolocalised to mucus producing cells (Artis, 2006). The exact function is uncertain, however known early upregulation in the jejunum of *T. spiralis* infected mice would support a role in innate immunity (Knight et al., 2004). The following is a review of the members of this recently discovered gene family.

1.7.2 RELM/FIZZ family

This family of novel class of cysteine rich tissue specific secreted proteins have been independently termed resistin like molecules (RELMs) and the found in inflammatory zone (FIZZ) family. The RELM/FIZZ family include four murine proteins: FIZZ1/RELMα, FIZZ2/RELMβ, FIZZ3/Resistin, RELMγ, and two human: FIZZ2/RELMβ and Resistin. The consensus RELM sequence is a protein of 104 - 114 amino acids with three domains: an N-terminal signal sequence, a variable

middle portion and a highly conserved C-terminal sequence (Steppan et al., 2001b). There is a unique cysteine repeat motif C-X_{11}-C-X_8-C-X-C-X_3-C-X_{10}-C-X-C-X-C-X_9-CC-X_{3-6}-END. Members of this family have been shown to form homodimers and heterodimers with the exception of FIZZ1 (Banerjee and Lazar, 2001). On recent analysis of their crystal structures FIZZ2 and FIZZ3 were shown to have a unique multimeric structure (Patel et al., 2004).

1.7.3 Murine FIZZ1/ RELMα/HIMF/PMNG1

FIZZ1 was identified in a screen for novel secreted proteins in bronchoalveolar lavage fluid of mice with experimentally induced allergic pulmonary inflammatory disease (Holcomb et al., 2000). Expression was immunolocalised to bronchial epithelial cells and non-neuronal cells adjacent to neurons in the peribronchial stroma in control animals. There was marked increased in expression in the lung epithelial mucosal cells and de novo expression in type II alveolar pneumocytes in allergen challenged animals. Expression was also demonstrated in the submucosa of the gut in cells adjacent to nerve fibres and in scattered cells in the subcutaneous tissue of the mammary glands. In functional studies FIZZ1 was found to modulate nerve growth factor (NGF) mediated embryonic dorsal root ganglion (DRG) survival and gene expression induced by NGF in adult DRG neurons (Holcomb et al., 2000). These findings were suggestive of a role for FIZZ1 in modulation of neuronal activity in the respiratory tract. Interestingly NGF has been implicated as an amplifier of Th2 effector function (Braun et al., 1998).

FIZZ1 has also been termed hypoxia induced mitogenic factor (HIMF) because of its discovery in a cDNA micro-array analysis of a mouse chronic hypoxia model, in a search for novel hypoxia inducible genes in the lung (Teng et al., 2003). Expression was immunolocalised to the pulmonary vascalature, bronchial epithelial cells and type 11 pneumocytes. *In vitro* studies showed that HIMF stimulated pulmonary microvascular smooth muscle cell proliferation and increased pulmonary arterial pressure and vascular resistance more potently than either endothelin-1 or angiotensin-1.

FIZZ1 has also been termed RELMα (Steppan et al., 2001b) and expression shown in murine adipose tissue, mammary tissue, heart, lung and tongue. It was found to be down regulated in white adipose tissue from diabetic mice, and partially normalised by treatment with the beta 3 agonist BRL-35135, suggesting that it may play a role in adipocyte homeostasis (Moore et al., 2001). FIZZ1 has been demonstrated in alternatively activated macrophages (Loke et al., 2002), and expression has also been shown in other antigen presenting cells (Nair et al., 2006) and in an experimental model of murine trypanosomosis *in vivo* induction of expression was shown to occur in an IL-4 dependent manner (Raes et al., 2002). In another study using a mouse model of allergic inflammation FIZZ1 was shown to be upregulated in the lungs, three hours after antigen challenge, in a STAT6 dependent manner (Stutz et al., 2003). The promoter region of FIZZ1 was found to contain functional binding sites for STAT 6 and CCAAT/Enhancer binding protein (C/EBP) and the FIZZ1 promoter reporter gene constructs, responded to IL-4 and IL-13 stimulation in transiently infected cells.

FIZZ1 has been implicated in mediating the deposition of extra cellular matrix in a rat model of bleomycin (BLM) induced lung fibrosis, in myofibroblast differentiation (Liu et al., 2004a; Liu et al., 2004b) and recently an anti-apoptotic effect has been demonstrated in lung fibroblasts (Chung et al., 2007).

1.7.4 Human FIZZ1/RELMα

To date FIZZ1 has not been recognised in people.

1.7.5 Murine FIZZ2/ RELMβ

1.7.5.1 Expression of RELMβ

Expression of FIZZ2 was first described in the colon and to a lesser extent in small intestine (Holcomb et al., 2000) and this molecule was later referred to as RELMβ (Steppan et al., 2001b). FIZZ2/ RELMβ mRNA expression was found to be strongest

in the proliferating epithelia at the bases of the crypts (Holcomb et al., 2000; Steppan et al., 2001b). Whilst, the mRNA for RELMβ was only expressed strongly in the crypt epithelium, subsequent migration of goblet cells from the crypt to the surface epithelium, resulted in protein detection in the surface epithelium (He et al., 2003) Immunohistochemical studies showed that FIZZ2 appeared to be released from goblet cell granules into the lumen of the colon and isolation of protein from the stool of several mice showed that it was secreted at high levels in the stool (He et al., 2003). In min mice, which harbour a mutation of the APC gene causing familial adenomatous polyposis, FIZZ2 expression was markedly increased in tumour tissue (Steppan et al., 2001b). These findings suggest that FIZZ2 expression is increased in intestinal epithelial cells with increased proliferative rate regardless of the cause.

1.7.5.2 RELMβ and the microbial environment

The bacterial milieu of the colon appears to be associated with the expression of FIZZ2 (He et al., 2003). Studies in germ free mice have shown that FIZZ2 mRNA and protein expression are severely reduced or absent in germ free mice however exposure to a conventional environment for four days resulted in robust expression in colonic epithelium similar to conventional mice. A similar response was found in C.B17.SCID mice showing that this response is independent of the specific adaptive immune system. However recent work using SAMP1/Fc mice, which develop a spontaneous ileitis, has shown that upregulation of RELMβ occurs coincident with the onset of inflammation, however viable intestinal flora were not required for the induction of RELMβ or ileitis (Barnes et al., 2007).

1.7.5.3 RELMβ in obesity and glucose homeostasis

RELMβ appears to play a role in glucose homeostasis as studies in rats have shown that administration of RELMβ resulted in acute impairment of hepatic insulin sensitivity and glucose metabolism (Rajala et al., 2003). Furthermore serum levels have been shown to be higher in mice fed a high fat diet compared to those on a normal diet and were also higher in diabetic mice compared to lean litter mates (Shojima et al., 2005). In addition, transgenic mice which overexpress RELMβ in the

liver exhibit hyperglycemia, hyperlipidemia and fatty liver (Kushiyama et al., 2005). Using the human mucoid colonic carcinoma cell line (LS174T) recent work has shown that RELMβ expression can be regulated directly by glucose and saturated fatty acids such as stearic acid as well as by hormones including insulin and TNFα (Fujio et al., 2007).

1.7.5.4 RELMβ and parasitic infections

In murine models RELMβ expression can be induced by nematode parasites that induce a typical TH2 response (Artis, 2006). Furthermore in cultures using the human colonic cell line (LS174T) RELMβ expression was induced when either recombinant IL-4 or IL-13 was added to the medium and inhibited by interferon gamma. Studies using IL-4KO and IL-4Rα KO mice have shown that the mechanism of induction of RELMβ is IL-4 independent and IL-4Rα dependent (Artis et al., 2004). The presence of STAT6 binding sites in the RELMβ promoter (He et al., 2003) supports a role for IL-4 and IL-13 mediated STAT6 activation and induction of RELMβ. When recombinant IL-13 (rIL-13) was administered intra-peritoneally to *T. muris* infected AKR mice, which are normally susceptible to infection, there was a marked induction of RELMβ in the caecum and small intestine and a significant reduction in worm burden. In a study looking at the interaction of RELMβ with parasites, recombinant RELMβ was shown to bind to the bacillary band of *T. muris in vivo* and to alter the chemoattractant properties of *S. stercolis in vitro* suggesting a possible role for this protein in nematode chemosensory function (Artis et al., 2004).

1.7.5.5 RELMβ and the respiratory tract

When recombinant RELMβ was delivered intratracheally to naïve mice it induced dose dependent leucocyte accumulation, predominantly macrophages, and also goblet cell hyperplasia. In addition perivascular and peribronchial collagen deposition occurred. Whilst in an experimental model of allergic airway inflammation mice deficient in RELMβ were shown to have less collagen accumulation and less goblet cell hyperplasia. Together these findings are supportive

of an inflammatory and a remodelling role for RELMβ in the respiratory tract (Mishra et al., 2007).

1.7.5.6 RELMβ and macrophages

Of particular interest is a recent study which has demonstrated the ability of RELMβ to stimulate naïve bone marrow derived macrophages to secrete TNFα, IL6 and RANTES (regulated upon activation, normal T cell expressed and secreted) further support for a role for RELMβ in inflammation by inducing mucosal cells to produce proinflammatory cytokines (Barnes et al., 2007).

1.7.6 Human FIZZ2/RETNLB (resistin like beta)/RELMβ/HXCP2

Expression of FIZZ2 has been shown in the human colon and protein has also been detected in the stool (He et al., 2003). The function of this protein in humans is unknown.

1.7.7 Murine FIZZ3/Resistin/ adipose tissue specific secretory factor (ADSF)

FIZZ3 was first identified as a result of a cDNA microarray analysis of rat adipose tissue to identify novel genes specific to adipocytes and induced during adipocyte differentiation (Kim et al., 2001) and was termed adipose tissue specific secretory factor (ADSF). Its expression was found to be under tight nutritional and hormonal regulation. Expression in adipose tissue was found to be very low in streptozotocin treated diabetic mice and increased 23 fold upon insulin administration. Expression was also found to be low in starved mice and increased 25 fold after feeding a carbohydrate rich meal.

Simultaneously (Steppan et al., 2001a) described a unique signalling molecule secreted by mouse adipocytes which they termed resistin (resistance to insulin). Circulating levels of resistin were shown to be increased in diet induced and genetic forms of obesity and downregulated by the administration of thiazolidineiones, and

peroxisome proliferator activated receptor gamma agonists (Steppan et al., 2001a) (Olefsky and Saltiel, 2000). Insulin stimulated glucose uptake has been shown to be enhanced by neutralization of resistin and reduced by treatment with recombinant resistin (Steppan et al., 2001a). More recently (Banerjee et al., 2004) have shown that resistin knock out mice exhibit lower blood glucose with reduced hepatic glucose production. Hence there has been considerable interest in resistin as one of the important adipokines causing insulin resistance.

1.7.8 Human FIZZ3/RETN/ADSF/RETN1/Resistin

Obesity is a major risk factor for insulin resistance and type 2 diabetes mellitus and whilst it was hoped that FIZZ3/Resistin would provide the link between obesity and type II diabetes, expression of resistin in rodents does not appear to correlate with findings in humans. Clinical trials have shown that there is significantly more resistin in the serum of obese humans, however it does not appear to be a predictor of insulin resistance (Degawa-Yamauchi et al., 2003). Recent research has shown a major species difference in that resistin is predominantly secreted by macrophages in humans and adipocytes in rodents (Curat et al., 2006). However, there is some evidence from *in vitro* studies using cultured human adipocytes to show that recombinant human resistin may influence adipose metabolism by an effect on preadopocyte cell number and cell lipid content (Ort et al., 2005). Of particular interest is the recent finding that addition of recombinant human resistin protein to macrophages, either murine or human, resulted in increased excretion of the inflammatory cytokines TNFα and IL-12 (Silswal et al., 2005). Several studies have now shown resistin as a useful biomarker of inflammatory disease state in people including atherosclerosis, rheumatoid arthritis, chronic kidney disease, septic shock (Reilly et al., 2005; Senolt et al., 2007; Sunden-Cullberg et al., 2007; Yaturu et al., 2007). As obesity is considered a mild inflammatory state with marked infiltration of abdominal fat with macrophages, controversy still continues as to whether resistin has a role to play in the pathophysiology of type II diabetes mellitus in people.

1.7.9 Murine FIZZ4/RELMγ/XCP1

FIZZ4 was identified as a gene that was downregulated in the nasal epithelium and the lungs of rats exposed to cigarette smoke, a model of oxidative stress (Gerstmayer et al., 2003). Interestingly in the same model FIZZ1 was found to be upregulated in the lungs after exposure to smoke. Expression of FIZZ4 was also shown in the bone marrow, spleen, white blood cells, thymus and white adipose tissue of both rats and mice which temped one to speculate that specific immune cells are able to secrete FIZZ4 protein (Gerstmayer et al., 2003). FIZZ4 has also been shown to be expressed in the intestinal tract and in particular the colon, where it can form a heterodimer with FIZZ2 (Shojima et al., 2005). FIZZ4 is detectable in the serum and increased levels were found in mice fed a high fat diet and also in diabetic mice compared to lean litter mates (Shojima et al., 2005). The increased serum levels of FIZZ4 have been attributed to increased production in the colon and bone marrow. The discovery of FIZZ4 and its similarity to FIZZ1 has led to some concern regarding the specificity of some of the results of experiments regarding expression of FIZZ1, as antibodies to FIZZ1 may cross react with antibodies to FIZZ4.

1.7.10 Human FIZZ4

To date all searches for human orthologs of FIZZ4 have been unsuccessful.

1.8 Hypothesis

The mucus associated molecules ITLN, SIAT4C and RELMβ had been shown in murine models to be upregulated in infection with parasites known to induce a Th2 response. In the present work, it was hypothesised that these three mucus-associated molecules were co-regulated by Th2 cytokines and that their upregulation was part of a typical anti-parasite response in other species.

1.9 Aims of this project

- To use *in vitro* cell culture techniques with human cell lines to determine if there was co-regulation of ITLN, SIAT4C and RELMβ transcripts in response to incubation with Th2 cytokines

- To use *in vitro* cell cultures techniques with human cell lines to determine the effect of pro-inflammatory cytokines on the expression of SIAT4C

- To clone and sequence sheep ITLNs and SIAT4C and determine tissue distribution in this species. RELMβ did not appear to be expressed in sheep.

- To examine the expression and co-regulation of SIAT4C and ITLN transcripts in a Th2 type environment in the respiratory and gastro-intestinal tract of sheep

- To determine if either SIAT4C or ITLNs had a role to play in protection against parasitic disease in sheep

2 MATERIALS AND METHODS

2.1 Cell lines and animals

2.1.1 Cell lines

Two mucoid cell lines were used; LS174T, a human colonic mucoid adenocarcinoma cell line and NCI-H292, a human respiratory mucoepidermoid carcinoma cell line (The European collection of Animal Cell Cultures, Porton Down, UK.)

2.1.2 Sheep for harvesting tracheal explants

Sheep tracheal explants were harvested from freshly culled aged ewes in the Department of Pathology at the University of Edinburgh.

2.1.3 Sheep and lambs for the Moredun Research Institute *T. circumcincta* infection experiments

Scottish Blackface x Leicester or Dorset x Suffolk lambs (5 months) and yearlings (8-12 months) were born and maintained at the Moredun Research Institute (MRI), under conditions designed to exclude accidental infection with nematode parasites. Male and female animals were included. All experiments were approved by the Institute's ethical review committee and were performed under licence, as required by the United Kingdom's Animals (Scientific Procedures) Act of 1986.

2.1.4 Infective larvae for the Moredun Research Institute *T. circumcincta* infection experiments

Infective third stage larvae from an anthelmintic susceptible isolate of *T. circumcincta* were isolated and stored at 4°C for up to a month before use. All the challenge doses used within each experiment were from the same batch of larvae.

2.1.5 Lungworm infected sheep and controls

The lungworm infected sheep were yearlings, from a Scottish black face commercial flock naturally infected with *Dictyocaulus filarial*; infection had been confirmed by faecal analysis using the Baermann technique. The control sheep were Dorset cross Suffolk yearling sheep born and maintained at the MRI under conditions designed to exclude accidental infection with nematode parasites.

2.1.6 Lambs for the University of Edinburgh *T. circumcincta* infection experiment

Forty eight Blackface dams were crossed with three Blackface sires to give birth to a number of lambs from which 57 females were chosen. Dams and sires belonged to a Scottish Blackface sheep population previously used for quantitative genetic and quantitative trait loci (QTL) analyses of faecal egg counts (Davies et al., 2006). In order to maximize genetic variation in parasite resistance, dams were selected from the extremes of the distribution of their estimated breeding values for faecal egg count (FEC). The three sires were chosen for having average estimated breeding value for FEC and each were mated with an equal number of dams from the top-half and bottom-half of the breeding value distribution. Sheep and lambs were owned by the University of Edinburgh (UoE) and maintained at the Roslin institute (Scotland, UK). All experiments were performed under licence, as required by the United Kingdom's Animals (Scientific Procedures) Act of 1986.

2.2 Experimental Protocols

2.2.1 Human cell line culture protocol

LS174T cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco/Invitrogen, Paisley, UK) supplemented with non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco/Invitrogen, Paisley, UK), 2 mmol L-glutamine (Gibco/Invitrogen, Paisley, UK) and 10% heat inactivated foetal calf serum (Gibco/Invitrogen, Paisley, UK). NCI-H292 cells were kept in RPMI 1640 with NaHCO2 without L-Glutamine (Sigma-Aldrich, Paisley, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco/Invitrogen, Paisley, UK), 2 mmol L-glutamine (Gibco/Invitrogen, Paisley, UK) and 10% heat inactivated foetal calf serum (Gibco/Invitrogen, Paisley, UK). Each new batch of supplemented medium was checked for sterility by incubating 1ml of medium with 1 ml of nutrient broth for 24 h at 37°C with 5% CO_2.

The cells were initially cultured in 75 mm^3 tissue culture flasks in an incubator at 37°C with 5% CO^2, and once cells became confluent, they were seeded into 25 mm^3 flasks with 2 x 10^5 cells/ml. To remove cells from the 75 mm^3 flasks, the culture medium was decanted and the cells were rinsed with 6 mls of versene (ethylene diamine tetraacetic acid) which chelates calcium and magnesium. The versene was removed and replaced by 5 ml of trypsin which detaches cells by digesting the proteins that keep the cells attached, the time varied from 5-10 min and incubation at 37°C was necessary for the NCI-H292 cells. Versene increases the efficacy of trypsin. Once cells had detached 10 ml of culture medium were added and cells transferred to a 50 ml centrifugation tube and centrifuged at 1250 rpm for 5 min. The supernatant was removed and cells resuspended in 10 ml of medium. Cell counts were done manually using a haemacytometer and viability was checked using 0.2% tryptan blue before cells were seeded into 25 mm^3 culture flasks.

Recombinant human interleukin-4 (IL-4), IL-9, IL-13, IFNγ, TNFα (Peprotech EC, London, UK) were made up according to the manafacturer's recommendation and used at a concentration of 1 or 10 ng/ml depending on the experiment. Cytokines

were added directly to the medium initially. Then cells were added at 2 x 10^5 cells/ml to a total volume of 10 ml. Flasks were set up in fours and control flasks were set up containing medium only. Flasks were checked daily for signs of contamination and confluence and were harvested at 12, 24 or 48 h depending on the experiment.

Cells were loosened using disposable cell scrapers (Fisher Scientific, Loughborough, UK) and transferred into 15 ml tubes. Following centrifugation at 1200 rpm for 5 min, the supernatant was discarded and cells resuspended in 1ml of TRI Reagent (Sigma-Aldrich, Irvine, UK) before storage at -70°C.

2.2.2 Culture of LS174T cells for Phalloidin/Sytox green immunolabelling

A sterile coverslip was placed in each well of a 6 well culture trays. Cells were seeded at 2 x 10^5 cells/ml either in medium or medium containing IL-4 (10 ng/ml). They were incubated for 48 h and checked for contamination and morphological changes initially every 2 h then every 12 h. The supernatant was removed and 4% paraformaldehyde put in each chamber for 15 min. This was decanted and cells washed twice with PBS pH 7.4 prior to immunolabelling.

2.2.3 Protocol for culture of tracheal explants

The culture technique was a modification of previously published techniques (Kitson et al., 1999; Lin et al., 2001) and has recently been published (McNeilly et al., 2007). Tracheal rings were harvested from freshly killed sheep, washed 3 times in phosphate buffered saline (PBS) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml amphotericin B and 10 µg/ml gentamycin. Following removal of excess fat, equal sized discs of tracheal mucosa were dissected from the underlying cartilage using a 6 mm sterile punch biopsy (Kruuse, A/S, Marsley, Denmark) and placed epithelial surface upwards into the bottom of 6.5 mm diameter, 0.4 µm pore size Transwell® cell culture inserts (Corning Life Sciences, Acton, USA) within 24 well tissue culture plates. Mucosal explants in triplicates were cultured in an air-liquid interface for 48 h at 37^0C with 5% CO_2 in Dulbecco's

modified Eagle's medium (Invitrogen) containing 2% ultraserosa G serum substitute (Ciphergen, Fremont, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml amphotericin, 10 µg/ml gentamycin and 100 U/ml insulin with or without recombinant sheep IL-4. Recombinant sheep IL-4 was expressed in Chinese hamster ovary (CHO) cells as previously described (Hope et al., 2005) and the resulting supernatant was used at 1:100 dilution. Explants were harvested at 48 h and fixed in 4% paraformaldehyde for histopathology, immunohistochemistry, carbohydrate histochemistry or placed in RNA later (Qiagen) for RNA extraction. Samples in 4% paraformaldehyde were submitted to the Division of Veterinary Pathology at the R(D)SVS for paraffin embedding, cutting of 4 µm sections and mounting onto coated glass slides (Snowcoat X-tra; Surgipath, Winnipeg, Manitoba, Canada). Triplicate samples were embedded together. Tissues in RNA later were stored at -20°C.

2.2.4 Protocol for University of Edinburgh *T. circumcincta* infection experiment

Ten lambs were chosen as controls and were bred and maintained in a nematode-free fenced area. The remaining 47 lambs, were maintained on the same farm as the controls but in a separate fenced area, and were experimentally infected with approximately 2,300 infective larvae three times a week until killed. Control lambs were sham dosed at the same time. Experimental protocol is summarised in Table 2.1.

Groups	Trickle [a] infection	Number killed
Trickle infected	+	47
Controls	-	10

Table 2.1: Experimental protocol for University of Edinburgh *T. circumcincta* infection experiment

[a] 2300 *T.circumcincta* L3, 3 times per wk for approximately 16 weeks.

2.2.5 Protocol for Moredun Research Institute *T. circumcincta* infection experiments

Using a similar protocol experiments were conducted over three successive years (2004-2007). For the purpose of this thesis, experiments have been grouped into four trials, numbered in chronological order, the designs of which are summarised in Table 2.2. For descriptive purposes sheep were divided into the following four groups; group 1: naïve unchallenged (unv); group 2: naïve challenged (cnv) killed at set time points post challenge; group 3: previously infected unchallenged (upi) and group 4: previously infected challenged (cpi) killed at set time points post challenge. The previously infected animals were given 2000 stage 3 larvae (L3) three times per week for eight weeks, a protocol which has previously been shown to confer some immunity (Smith et al., 1983a) All animals were treated with anthelmintic (Levamisole) prior to challenge to clear any residual worms in the previously infected group, and as a control treatment in the other groups. In total 75 yearling sheep were included in trials 1-3, and 35 lambs in trial 4.

Trial	Sheep	Status	Treatments *(relative to day of challenge)*						
			Trickle[a] infection	Levamisole[b]	Number killed				
					Unchallenged		Challenged[c]		
(Day)				*(- 7)*	*(0)*	*(2)*	*(5)*	*(10)*	*(21)*
1	Yearling	Previously infected	+	+				6[e]	
		Naïve	-	+				6[e]	3[d]
2	Yearling	Previously infected	+	+			6[d]	6[d]	
		Naïve	-	+	6[d]		6[d]	6[d]	6[d]
3	Yearling	Previously Infected	+	+	6[e]	6[e]			
		Naïve	-	+	6[e]	6[e]			
4	Lamb	Previously Infected	+	+			6[e]	5[e]	5[e]
		Naïve	-	+	4[e]		6[e]	5[e]	4[e]

Table 2.2: Experimental protocol for the Moredun Research Institute *T. circumcincta* infection experiments

[a] 2000 *T. circumcincta* L3 3 x per week for 8 weeks, [b] Levamisole 7.5mg/kg per os, [c] 1 x 50,000 *T. circumcincta* L3, [d] Dorset x Suffolk, [e] Scottish Blackface x Leicester.

2.2.6 Post mortem procedure

Sheep were killed either by captive bolt or by intravenous injection of pentobarbitone sodium (Merial Animal Health Ltd, Harlow, UK).

2.2.7 Worm counts from *T. circumcincta* infected sheep

The worm counting technique used in sheep from the MRI has been published (Halliday et al., 2007). In brief the abomasal contents were collected and the abomasum washed twice in warm saline. The washings were added to the contents. The abomasum was then placed in

saline at 37°C for 4 h to recover larvae in the mucosa. The abomasal digests and contents were made up separately to 5 l and fixed in 2% formalin. Following mixing a 5% sub-sample was obtained, quantified and stained with iodine. Total number of worms was calculated, in addition sex and worm stage were noted. For University of Edinburgh (UoE) sheep the methodology differed slightly. The abomasum content was made up to a volume of 10 l. Following thorough mixing, a 10% aliquot was removed and passed through a 38 mm sieve. All gastrointestinal nematodes in a 1% aliquot were stained with iodine solution and identified to developmental stage, species and sex and the tallies multiplied by 100 to obtain an estimate of total burden. Abomasal mucosae were subject to peptic digestion for the recovery of arrested larvae as outlined by MAFF (1986).

2.2.8 Tissue collection from *T. circumcincta* infected sheep

For tissue analysis from *T. circumcincta* infected sheep (MRI and UoE) the gastric lymph nodes and the abomasum were isolated and removed. The abomasum was bisected longitudinally and one half retained for worm counting along with the abomasal contents. Multiple small pieces of tissue (approximately) 1cm x 1cm were collected from the fundic fold of the other half of the abomasum and from the gastric lymph nodes immediately post mortem and placed in either 4% paraformaldehyde for histopathology sections, RNA later (Qiagen, Hilden, Germany) for RNA isolation or snap frozen for protein extraction. Tissues in RNA later were stored at -20°C. After 24 h fixation samples in 4% paraformaldehyde were transferred to 70% ethanol and were submitted to the Division of Veterinary Pathology at the R(D)SVS for paraffin embedding, cutting of 4 µm sections and mounting onto coated glass slides (Snowcoat X-tra; Surgipath, Winnipeg, Manitoba, Canada).

2.2.9 Broncho-alveolar lavage of normal sheep

Bronchoalveolar lavage (BAL) samples were collected from MRI Trial 2 group 1 sheep at post mortem prior to collection of tissue for immunohistochemistry and RNA extraction. Two 30 ml aliquots of sterile saline were instilled into the left main stem bronchus using a 50 ml syringe and withdrawn using gentle suction, samples were pooled. Cytospins were prepared by centrifugation of 100 µl of BAL fluid at 300g for 3 min (Shandon Cytospin 3). Cytospins were air dried prior to staining with

Leishman's stain (Fisher Scientific). Two hundred cells were counted and differential counts calculated.

2.2.10 Collection of tissues from normal sheep: respiratory tissue and multiple tissues

For normal sheep respiratory tract, multiple small pieces of tissue (approximately) 1cm x 1cm were collected from the tracheal mucosa and the left caudal lung lobe from MRI Trial 2 group 1 sheep. For evaluation of multiple tissues small pieces of tissue (approximately) 1cm x 1cm samples were collected from tongue, jejunum, colon, Peyer's patches (ileum), mesenteric lymph node, rectum, lung, liver, kidney, spleen, pancreas, heart, skin, ovary, uterus, fat and brain of MRI Trial 3 group 1 sheep. Tissues were processed and stored as described previously.

2.3 Molecular biology techniques

2.3.1 RNA extraction

2.3.1.1 RNA extraction from cultured cells

Cells stored in TRI Reagent were thawed and transferred into 1.5 ml eppendorf tubes. 200 µl of chloroform ((Sigma-Aldrich, Paisley, UK) was added and samples were vortexed for 30 sec. They were then left to stand for 5 min before centrifugation at 13,000 rpm for 15 min. The top clear phase was decanted into a 1.5 ml eppendorf, isopropanol (500 µl) was added to precipitate the RNA and after thorough mixing the sample was left to stand for 10 min. Precipitated RNA was pelleted by centrifugation at 13,000 rpm for 10 min. The pellet was washed with 70% ethanol by vortexing for 5-10 sec and following centrifugation at 8,000 rpm for 5 min the supernant was removed and the RNA pellet air dried, prior to dissolving in 50 µl of RNase free water, by incubating at 60°C for 15 min. RNA was stored at -70°C.

2.3.1.2 RNA extraction from sheep tissues

Tissue was prepared for RNA extraction using the Stratech Beadbeater-8 (Stratech Scientific, Soham, UK) and Qiashredder (Qiagen, Crawley, UK) and total RNA was extracted using the RNeasy kit (Qiagen) as per the manufacturer's instructions. Approximately 30 mg of tissue was cut into small pieces, placed in tubes prefilled with $1mm^3$ Zirconia/silica beads (Thistle Scientific, Glasgow, Scotland, UK) and 1ml of RLT buffer (with beta mercaptoethanol added) and spun for 4 x 1 min in the Mini-beadbeater (Biospec products). The tissue lysate was collected and passed through a Qiashredder column by centrifugation at 13,000 rpm for 2 min to further disrupt tissue. The flow through was harvested and following addition of 70% ethanol RNA was loaded onto the silica membrane of the RNeasy column by centrifugation at 13,000 rpm for 1 min. RNA was washed with buffer RW1 and on column DNA digestion performed by incubation with 80 µl DNase 1 (RNase-free DNase Set, Qiagen) for 15 min at room temperature. DNA free RNA was washed with buffers RW1 and RPE, eluted in 200 µl of RNase free water and quality and quantity measured by spectrophotometry.

2.3.2 RNA quantification and quality

Quantity, quality and purity of RNA were assessed using either the Beckman DU 650 or Cecil 2021 2000 series spectrophotometers. Following calibration with RNase free water a diluted RNA sample was loaded into the cuvette. The spectrophotometer measures optical densities (OD) at different wavelengths of UV light. The OD at 260 nm can be used to calculate the quantity of RNA by using the following formula:

RNA (µg/ml) = (OD260) x (40*) x (100**)

* 40 used for RNA, 50 for DNA, 33 for cDNA. ** Dilution factor.

The dilution factor was optimised to ensure that the recorded optical density at 260 nm was between 0.1 and 1.0 which is considered to give the most accurate recording. The ratio of OD at 260 nm to 280 nm gives a measure of the purity of a sample as the

OD at a wavelength of 280nm is the measure of the amount of protein in a sample. The ratio should be between 1.6 and 2.0.

Selected RNA samples were submitted to the Roslin Institute for further RNA quality assessment using the BioAgilent system prior to full length ITLN product amplification using the BD Smart Race cDNA amplification kit (BD Biosciences, Oxford, UK). The RNA Integrity number (RIN) is a measure of quality and should be between 6 and 10, only samples greater than 8 were selected for use in full product amplification.

2.3.3 DNAse treatment

For RNA extracted from cell cultures contaminating DNA was removed prior to reverse transcription using the Ambion DNA free kit (Ambion, Huntingdon, UK). In a 500 µl eppendorf, a 5 µg sample of RNA was made up to 41 µl using RNase free water and 4 µl of DNase 1 enzyme and 5 µl DNase 1 buffer were added to make a total volume of 50 µl. This was incubated in a waterbath at 37°C for 1 h. 10 µl of resuspended DNase inactivation reagent was added, mixed and left to stand for 2 min before centrifugation at 13,000 rpm for 1 min to pellet the inactivation reagent. The samples were stored at -70°C. A separate DNase treatment was not required for any of the sheep RNA as the RNeasy kit (Qiagen) includes a DNase treatment step.

2.3.4 Reverse transcription of mRNA

DNA free mRNA was reverse transcribed using oligo dT primers and avian myeloblastosis virus (AMV) reverse transcriptase using the standard protocol according to the manafacturer's instructions (Reverse transcription system, Promega, Southampton, UK). A mastermix was made to increase accuracy of pipetting. The mastermix included 4 µl of 25 mM MgCl2 , 2 µl of 10 x buffer (100 mM Tris HCl pH 8.8), 2 µl of 10 mM dNTP mix, 0.5 µl of RNAse inhibitor, 0.5 µl AMV reverse transcriptase and 1 µl oligo dT primers per sample. 10 µl of mastermix was added to 10 µl (1µg) of RNA in a 500 µl eppendorf and incubated at 42°C for 1 h followed by 5 min at 99°C to denature the enzymes and then cooled to 4°C. All steps were done

in a Techgene PCR machine (Techne, Cambridge, UK). The samples were diluted to 100 µl and stored at -20°C.

2.3.5 RT-PCR for transcript expression in tissues

All reactions were carried out using either GeneAmp 2400 PCR machine (Perkin Elmer, Applied Biosystems, UK) or Touchgene Gradient 96 well machine (Techne, Cambridge, UK). A mastermix was made to increase pipetting accuracy which included 5 µl of 10 x buffer (100 mM Tris HCl, 15 mM MgCl2), 10 µl of either 2 or 10 µM primers (equal mix of sense and anti-sense primers), 0.5 µl Taq polymerase (5 u/µl) (Roche Diagnostics, GmbH, Mannheim, Germany), 24.5 µl RNase free water and 5 µl of 2 mM dNTPs (Promega, Southampton, UK) per sample. 45 µl of the mastermix was placed in 200 µl Perkin Elmer tubes (Axygen Scientific, Union City, CA, USA) and 5 µl (50 ng) of cDNA added. Reactions were carried out using the recommended conditions for the primers if known, otherwise an annealing temperature was selected that was a few degrees below the recommended annealing temperature of the primers and degradation and extension conditions were selected based on protocols for other primers. RNA only controls (0.5 µg RNA in 50 µl nuclease free water) were used to check for native DNA contamination.

2.3.6 Visualisation of PCR products on agarose gels

PCR products were mixed with loading dye at a 1:5 ratio and were electrophoresed on an agarose gel with 0.1% ethidium bromide. The concentration of the agarose gel (1.2%, 1.4%, 1.6%), the duration of electrophoresis (30 or 45 min) and the voltage used (100 or 140 volts) depended on expected PCR product size. Gels were photographed using a Kodak digital science image station 440CF or Bio-Rad FX imager (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK)

2.3.7 RT - PCR optimisation for semi-quantitative analysis

The number of thermocycles for semi-quantitative RT-PCR was determined by amplification of PCR products from each gene of interest at a range of cycle times

from 18-39. The PCR products were sunbjected to electrophoresis on an agarose gel with 0.1% ethidium bromide and the net intensity of the bands visualised was measured using the Kodak ID image analysis software. Using GraphPad Prism (GraphPad Software, San Diego, CA, USA) results were plotted on a graph and for each gene a thermocycle number selected that correlated with the amplification stage of the PCR reaction (Figure 2.1).

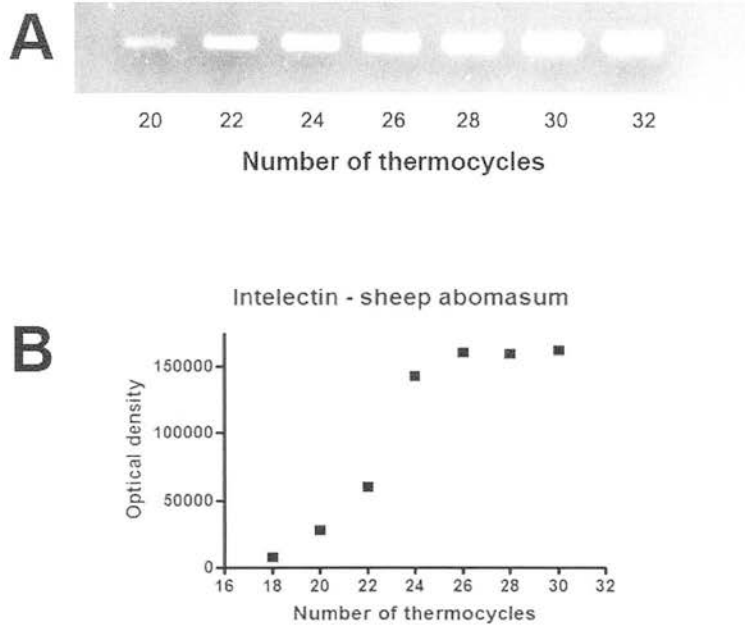


Figure 2.1: Standard curve for semi - quantitative RT-PCR

(A) 1.6% ethidium bromide gel showing intelectin PCR product at different thermocycles. (B) Standard curve to identify amplification phase of PCR reaction for semi-quantitative RT-PCR produced from optical density of bands shown in (A) plotted against number of thermocycles.

2.3.8 Semi-quantitative RT-PCR analysis

The density of each band visualised on agarose gels was calculated using the 1D Analysis software supplied with the Kodak® Digital Science Image Station 440CF. Transcript expression was normalised to a house keeping gene by calculating a ratio of the optical density of the transcript of interest compared to the house keeping gene

using Microsoft Excel software 2003. Every effort was made to use house keeping genes that demonstrated the least variance in the selected experimental conditions.

2.3.9 Quantitative reverse transcriptase real time polymerase chain reaction qPCR

Quantitative RT-PCR was performed using an Opticon 1 real-time PCR machine (MJ Research, GRI, Rayne, UK) and Quantitect SYBR Green (Qiagen) as the fluorescent probe. In order to generate a standard curve a PCR product containing the target sequence was amplified using external primers for the gene of interest. Six different concentrations of the standard were used to generate the standard curve ensuring the target fell within the range tested. 200 ng of cDNA was amplified using 0.3 μM primers in 20 μl Sybr green mastermix (2.5 mM $MgCl_2$), (Qiagen). PCR conditions were as follows: 95°C for 15 min, then 50 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. The following primers were used; sITLN ext GSP 1 and 2, sITLN int GSP 1 and 2. Reactions were carried out in triplicate. Melting curves were calculated for each reaction to confirm the identity of the PCR product and to ensure primer–dimer formation did not occur. Master mix only controls were included in all reactions as blanks to check for contamination. Cycle thresholds were calculated using Opticon 1 software, correcting fluorescent values against blanks. Copy number was calculated using the following formula: Y molecules/μl = (X g/μl DNA/ (330 x 660)) x 6.022 x 10^{23}. Subsequent analysis was carried out using Microsoft Excel 2003 and GraphPad Prism4 (GraphPad Software, San Diego, CA, USA).

2.3.10 PCR product purification prior to sequencing

A PCR product purification step (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manafacturer's recommendations prior to submitting samples for sequencing. 500 μl of buffer was added to 100 μl of PCR product, which was then placed in a column and centrifuged at 13,000 rpm for 30 sec. The cDNA on the membrane was rinsed twice with wash buffer and eluted using 25 μl of

elution buffer. Products were subjected to electrophoresis and imaged as described previously (2.3.6) prior to submitting for sequencing.

2.3.11 Analysis of sequencing results

All nucleotide sequences were determined using dideoxy chain termination cycle sequencing by the Functional Genomics Unit, Moredun Research Institute, Midlothian, UK. Forward and reverse sequences were requested. Sequences were received as .ABI files and Chromas lite V2 software (http://www.technelysium.com.au/chromasa_lite.html) was used to reverse and complement the reverse sequences and to convert all sequences into FASTA format. L Align (http://www.ch.embneet.org/software/lalign_form.html) was used to align forward and reverse sequences. Where there was poor alignment the chromatogram was checked and subjective assessments made on correct nucleotide sequence. Sequences were subjected to a blast search (http://www.ncbi.nlm.nih.gov/blast) to check that they were the correct product. Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw).

2.3.12 Partial sequences of sheep ITLN1 and sheep ITLN2

Consensus primers for conserved regions of human and mouse ITLNs (cITLN F1 and cITLN R2) designed by Alan Pemberton were used to amplify a partial ITLN sequence using abomasal cDNA from six MRI group 3, day 10, trial 1 *T. circumcincta* infected sheep. Forward and reverse sequences were aligned and final sequences clustered to give a consensus 307 bp partial sequence which was named sheep ITLN2 (sITLN2).

Using this partial sITLN2 sequence forward and reverse primers (external (Ext) gene specific primers (GSP) 2 and 1) were designed using the programme PRIMER3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi). These gene specific primers were used to amplify a partial ITLN sequence from respiratory tissue from six MRI group 1, trial 2 sheep. Forward and reverse sequences were aligned and final sequences clustered to give a consensus 220 bp partial sequence which was

named sheep ITLN1 (sITLN1). The sequence shared 94% homology with the sITLN2 partial sequence.

2.3.13 Sequencing of full length Sheep ITLN2

2.3.13.1 Primer design and cDNA amplification

The consensus sITLN2 partial sequence was used to design Internal (Int) gene specific primers (GSP) to use with the BD smart race cDNA amplification kit (BD Biosciences, Clontech, California, USA). Before using this kit all control samples provided by the manufacturers were processed and shown to produce products of the correct size (Figure 2.2).

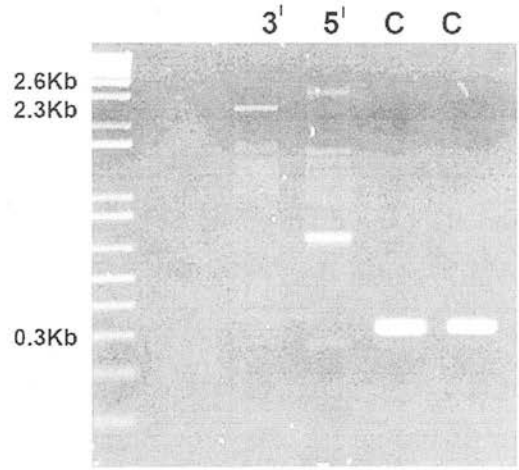


Figure 2.2: Amplification of control samples provided with BD smart race cDNA amplification kit.

Ethidium bromide gel showing PCR products for the 3' end, 5' end, and for positive control samples (C), all at the expected product size, 2.3Kb, 2.6Kb and 0.3Kb respectively. The 5' product was not seen at an initial annealing temperature of 68°C, however 65°C resulted in a visible band of the expected size.

A high quality (RIN 10) DNase treated abomasal RNA sample was selected that had shown strong expression of ITLN using the consensus primers cITLN F1 and cITLN R2. This sample was from sheep number A611, an MRI trial 1, group 4, day 10 sheep. In order to prepare the 5' race ready cDNA, the following reaction was set up

in a 500 µl Eppendorf on ice: 1 µg RNA, 1 µl 5'CDS primer, 1 µl BD Smart 11 A oligo + RNase free water to a total of 5 µl. The 3' race ready cDNA was prepared as follows: 1 µg RNA, 1 µl 3'CDS primer A and nuclease free water to a total of 5 µl. Contents of tubes were gently mixed and spun briefly before incubation at 72°C for 2 min. The samples were then reverse transcribed using BD powerscript reverse transcriptase in the following reaction: 2 µl 5 x 1st strand buffer, 1 µl DTT (20mM), 1 µl dNTP (10 mM) mix, 1 µl BD powerscript reverse transcriptase and incubated for 1 h 30 min at 42°C. Samples were diluted by adding 100 µl of tricene EDTA buffer, incubated for 7 min at 72°C and stored at -20°C. The BD smart 11 A oligo contains a terminal stretch of G residues and BD powerscript RT adds 3-5 residues (mainly dC) to the 3' end of the first strand cDNA which anneal to the BD smart 11 A oligo (Figure 2.3).

The 3' race ready cDNA was amplified using sheep ITLN external GSP2 primer, the BD advantage polymerase mix and touchdown PCR. Touchdown PCR increases specificity by cycling initially at a high temperature, hence the importance of designing suitable primers. The following 50 µl PCR reaction was set up on ice in 200 µl Perkin Elmer tubes; 5 µl 10 x BD advantage PCR buffer 2, 1 µl 10 mM dNTP mix, 1 µl 50 x BD advantage 2 polymerase mix and 34.5 µl nuclease free water, 2.5 µl 3' race ready cDNA, 1 µl universal primer mix, 1 µl sITLN ext GSP 1 10 µM. Positive and negative controls were also set up and all reactions were processed in the GeneAmp 2400 PCR machine (Perkin Elmer, Applied Biosystems, UK). PCR conditions were as follows: 94°C 2 min + 72°C 3 min x 5 cycles; 72°C 7 min + 94°C 2 min x 5 cycles; 94°C 30 sec, 65°C 30 sec, 72°C 3 min x 27 cycles initially, then a further 10 cycles.

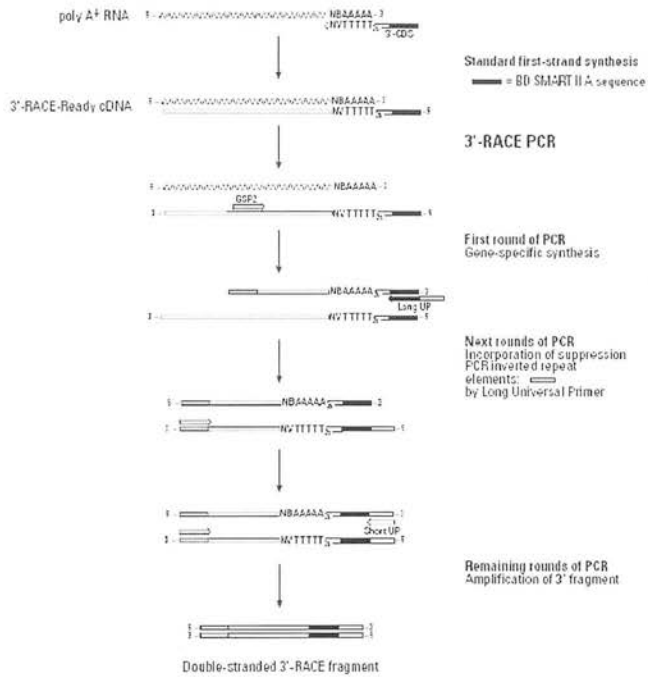
Amplification of the 5' required a different protocol which included a nested PCR reaction using GC melt and a polymerase designed for use with GC rich fragments. GC melt is used for amplification of products which are GC rich. The optimal concentration of GC melt varies with different primers, a trial reaction showed a concentration of 0.5 M gave best results. The following 50µl PCR reaction was set up on ice; 5 µl 10 x BD advantage PCR buffer 2, 1 µl 10 mM dNTP mix, 1 µl 50 x

BD advantage 2 polymerase mix and 34.5 µl nuclease free water, 2.5 µl 5' race ready cDNA, 5 µl universal primer mix, 1 µl sITLN ext GSP 1 10 µM. The reaction conditions were as follows: 35 cycles of 94^0C for 30 sec, 65°C for 30 sec and 72°C for 3 min. As only weak bands were detected on an agarose gel this was followed by a nested PCR for which the following was set up on ice: 10 µl BD advantage GC polymerase buffer, 5 µl 0.5 M GC melt (BD Biosciences, Clontech), 1 µl 10 mM dNTP mix, 1 µl BD Advantage™ GC 2 Polymerase (BD Biosciences, Clontech), 26 µl nuclease free water, 5 µl PCR product, 1 µl nested universal primer mix, 1 µl sITLN int GSP 1 10 µM. PCR products were run on a 1.2% agarose gel with 0.1% ethidium bromide, at 100 V for 60 min and visualised using the Kodak digital science image station 440CF. Details of primers supplied with BD smart race cDNA amplification kit (BD Biosciences) are shown in Table 2.3 and a flow chart of the BD smart race cDNA amplification technique is shown in Figure 2.3.

Primer name	Primer sequence
BD Smart 11 A oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3'-Race CDS primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30
5'-Race CDS primer	(T)25VN
10 x universal primer A long	CTAATACGACTCACTATAGGGCAAGCAGTGG TATCAACGCAGAGT
10 x universal primer A short	CTAATACGACTCACTATAGGGC

Table 2.3: Sequences of primers provided with the BD smart race cDNA amplification kit

A



B

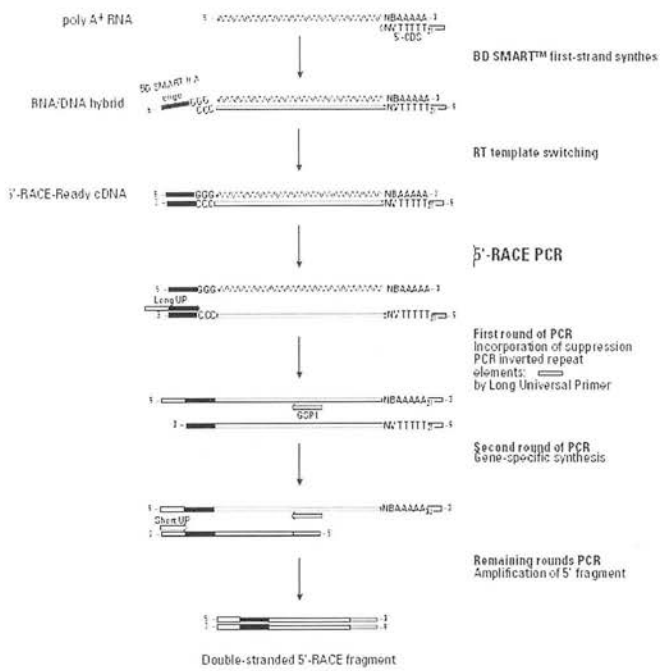


Figure 2.3: Detailed flow chart of BD smart race cDNA amplification

The flow charts show the method for amplification of double stranded 3' race fragment (A) and 5' race fragment (B) used in the sequencing of sITLN1 and sITLN2.

2.3.13.2 Ligation of PCR products into Plasmid Vector

PCR product from the 5' and 3' ends were ligated into the TA cloning vector pCR 2.1 (Invitrogen) in accordance with the manafacturer's instructions. Ligations were set up with 1 µl and 3 µl of fresh PCR product as follows; 1µl 10 x ligation buffer, 2µl pCR 2.1 vector (25 ng/ml), 1 µl T4 ligase, RNase free water (3 µl or 5 µl) and PCR 3' and 5' products (3 µl or 1 µl) to give total reaction of 10 µl. Ligations were incubated for 18 h at 14°C in a Techgene PCR machine (Techne, Cambridge, UK) and then stored at -20°C.

2.3.13.3 Transformation of competent cells

The ligated vector was used to transform INVαF' competent cells (Invitrogen). 2 µl of ligation reaction was added to one vial of thawed cells and gently stirred using pre-cooled pipette tips. The cells were incubated on ice for 30 min and then heat shocked for 30 sec at 42°C. 250 µl of SOC medium (Invitrogen) was added to each vial before incubation at 37°C for 1 h. The cell suspensions (20 µl and 200 µl) were plated out onto pre-warmed LB agar plates containing X-Gal (Promega) and 100 µg/ml ampicillin which were incubated overnight at 37°C. There was good growth of colonies, the colonies of transformed cells were white and the non-transformed cell colonies blue. Culture plates were stored at 4°C.

2.3.13.4 Isolation of plasmid DNA

White colonies were removed using sterile toothpicks and placed in 3 ml of liquid broth containing 100 µg/ml ampicillin and incubated overnight 37°C/200 rpm in a shaking incubator (Gallenkamp, Sanyo). There was evidence of good colony growth and plasmid DNA was isolated from broth using the Wizard plus SV Minipreps DNA purification system (Promega). Cells were pelleted by centrifugation at 13,000 rpm for 5 m and resuspended in 250 µl cell resuspension solution by vortexing. The cells were lysed by adding 250 µl cell lysis solution and then incubated for 5 min with 10 µl of alkaline phosphatase to inactivate endonucleases released during cell lysis. 350 µl of neutralisation solution was added and the lysate was centrifuged at 13,000 rpm for 15 min. The cleared lysate was placed in a spin column and plasmid

DNA bound to the filter by centrifuging at 13,000 rpm for 1 min. The DNA was washed twice by centrifugation at 13,000 rpm for 1 min with 750 µl and then 250 µl of wash solution. The filter was dried by centrifugation at 13,000 rpm for 2 min and DNA eluted by adding 100 µl of nuclease free water (Ambion) and centrifugation at 13,000 rpm for 1 min.

2.3.13.5 Restriction enzyme digest of Plasmid

The inserted DNA was cut out of the plasmid using the restriction enzyme Eco R1 (Roche). 2 µl of plasmid, 2 µl 10 x Buffer H containing Eco R1(Roche) and 16 µl of nuclease free water were set up on ice and 2µl of plasmid with 18 µl of RNase free water were set up as controls. All samples were incubated at 37°C for 2 h and then subjected to gel electophoresis on a 1.2% agarose gel containing 0.1% ethidium bromide at 100 V for 50 min and imaged as described previously (2.3.6). Samples with clear bands of the correct size (600-1000bp) were selected for sequencing.

2.3.13.6 Full sequence of sheep ITLN 2

Several clones were sequenced in both directions from both the 5' and 3' ends. Forward and reverse sequences of clones were aligned, the consensus sequences of clones were clustered, all Smart race and vector sequence was identified manually or by using EMVEC (http://www.ebi.ac.uk/blastall/vectors.html), then removed and the consensus 5' and 3' end sequences of sITLN2 identified. Using the overlapping 5' and 3' sequences the full sheep abomasal ITLN sequence was determined. Using primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi), primers was designed suitable for verification of the full sequence (sITLN F1 and sITLN R1) in abomasal mucosal samples of five sheep (MRI trial 1, group 4). The PCR products were purified (High Pure PCR Product Purification kit; Roche, Mannheim, Germany) as described previously (2.3.10), the sequences verified (http://www.ncbi.nlm.nih.gov/blast), the predicted open reading frame determined using ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf), and the predicted signal peptide and N-glycosylation sites identified using SignalP 3.0 (Http://www.cbs.dlu.dk/Services/SignalP) and NetNGlyc 1.0 Server

(Http://www.cbs.dlu.dk/Services/NetNGlyc/). Predicted glycophosphatidylinositol (GPI) anchor was determined using the programme GPI-SOM (http://genomics.unibe.ch/) (Fankhauser and Maser, 2005). Details of primers used in sequencing are shown in Figure 2.5 and Table 2.5.

2.3.14 Sequencing of full length sheep ITLN1

RNA samples were selected from normal sheep respiratory tissue (MRI trial 2, group 1 sheep numbers U5 and U6). These sheep were selected as they had shown strong expression of ITLN using the consensus primers cITLN F1 and cITLN R2. Using the forward primer sITLN 1 which had been used to sequence the full length sITLN2 with the reverse primer sheep ext GSP1 the 5' end of sITLN1 was amplified. Following temperature optimisation the following conditions were used: 94°C 2 min then 40 cycles of 72°C 40 sec, 55°C 40 sec, 72°C 2 min and a final extension at 72°C for 10 min. The PCR product was ligated into a vector, cloned and sequenced as described previously (2.3.13.2 - 2.3.13.6).

Amplification of the 3' end required preparation of 3' race ready cDNA using RNA from sheep U5. The 3' race ready cDNA was prepared on ice as follows: 254 ng RNA (3 µl), 1 µl 3'CDS primer A and nuclease free water to a total of 5 µl. Contents of tubes were gently mixed and spun briefly before incubation at 72°C for 2 min. The samples were then reverse transcribed using BD powerscript reverse transcriptase in the following reaction: 2 µl 5 x 1st strand buffer, 1 µl DTT (20mM), 1 µl dNTP (10mM) mix and 1 µl BD powerscript reverse transcriptase. Incubation was for 1 h 30 min at 42°C. Samples were diluted and stored as described previously (2.3.13.1).

Amplification of the 3' end required a nested PCR. The following 50µl PCR reaction was set up on ice; 5 µl 10 x BD advantage2 PCR buffer, 1 µl 10 mM dNTP mix, 1 µl 50 x BD advantage 2 polymerase mix and 34.5 µl nuclease free water, 2.5 µl 3' race ready cDNA, 5 µl universal primer mix, 1 µl sITLN ext GSP 2 10 µM. The reaction conditions were as follows: 25 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 3 min. As only smears were detected on an agarose gel this was

followed by a nested PCR for which the following was set up on ice: 5 µl BD advantage polymerase buffer, 1 µl 10 mM dNTP mix, 1 µl BD Advantage™ 2 Polymerase (BD Biosciences, Clontech), 34.5 µl nuclease free water, 2.5 µl PCR product, 5 µl nested universal primer mix, 1 µl sITLN int GSP 2 10 µM. Following temperature optimisation the reaction conditions were as follows: 20 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 3 min and a final extension of 72°C for 7 min. PCR products were visualised, ligated, cloned and sequenced as described previously (2.3.13.2 – 2.3.13.6)

The full sequence of sITLN1 was obtained from the overlapping sequences of the 5' and 3' ends and was verified in lungs from three sheep using the full length primers sITLN F3R3 designed using the primer 3 programme. The predicted ORF, N-glycosylation sites, signal peptide and GPI anchor were identified as described previously (2.3.13.6). Location of primers and details of primers used in sequencing are shown in Figure 2.4 and Table 2.5.

2.3.15 Sequencing of sheep ITLN3

2.3.15.1 Sequencing of partial sequence of sheep ITLN3

As part of the the DEFRA/SHEFC Veterinary Training Research Initiative (VT0102) a cDNA library was created using abomasal fold and lymph node tissue obtained from six MRI Trial 1 sheep. Data from this library was available in August 2006 and a search resulted in identification of two 3' end partial sequences of sheep ITLN from gastric lymph node, clones 9484 (510bp) and 9264 (470bp). Clone 9484 shared 98% homology with sITLN2 and 86% with sITLN1 whilst clone 9264 shared 93% homology with sITLN2 and 85% with sITLN1 and was considered to be a third sheep ITLN. Verification of this partial sequence of sITLN3 required design of primers that would differentiate the three sheep ITLNs.

2.3.15.2 Design of primers to differentiate sITLN1, sITLN2 and sITLN3

sITLN1, 2 and 3 specific reverse primers (sITLN R9-1, sITLN R9-2, sITLN R9-3) were designed for a poorly conserved region at the 3' end of the sequences and this was used with a consensus forward primer (sITLN F10) to amplify a 287 bp partial

sequence of each specific sheep ITLN (Figure 2.4). Specificity was confirmed by amplification of the product using RT-PCR in abomasal mucosa from MRI *T. circumcincta* infected sheep (Trial 2, group 4 sheep) followed by purification and sequencing as described previously (2.3.10 – 2.3.11). Homology of the three partial sequences with the three sheep ITLN sequences was determined using the programme ClustalW (Table 2.4).

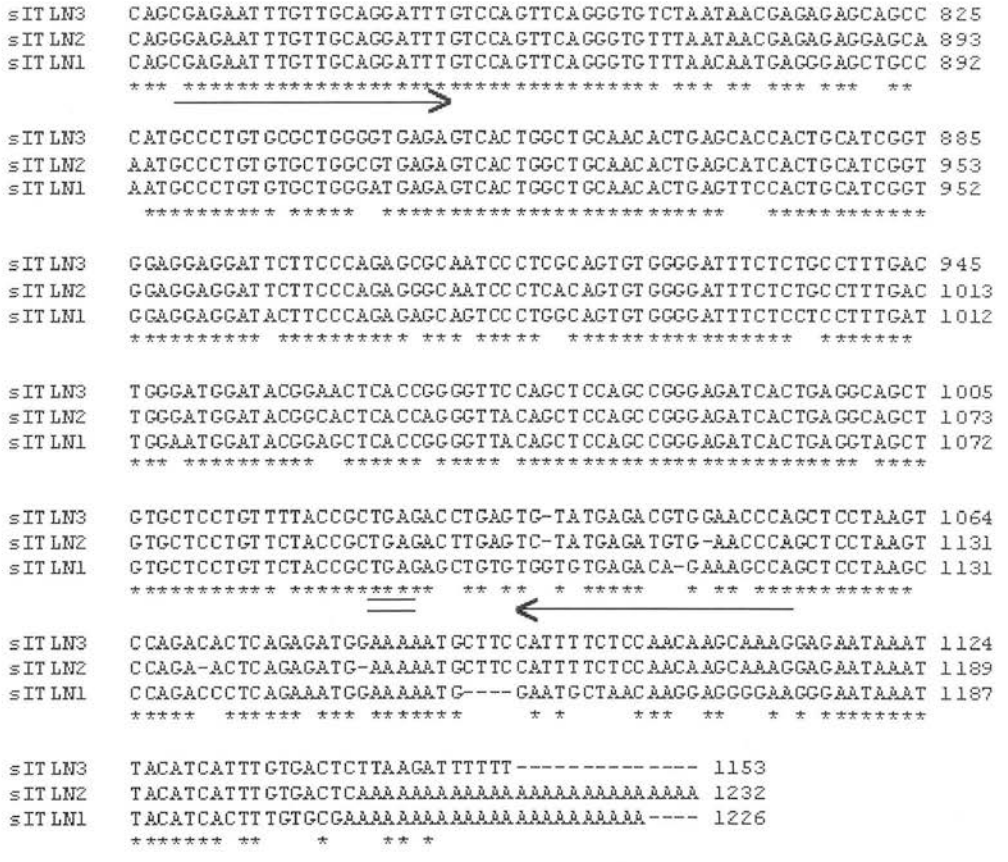


Figure 2.4: Alignment of 3' ends of sITLN1, sITLN2 and sITLN3

Alignment of the 3' ends of sITLN1, 2 and 3 showing location of primers used to differentiate the three sheep ITLNs. Forward and reverse primer location is marked by solid arrowed lines, indicating the direction of the primers. A double underline marks the stop codon of the proposed open reading frame. Asterix beneath aligned sequences represents conserved homology.

	sITLN1	sITLN2	sITLN3
sITLNF10 + R9-1	99%	88%	88%
sITLNF10 + R9-2	89%	98%	93%
sITLNF10 + R9-3	89%	93%	99%

Table 2.4: Homology of three partial sequences of sheep ITLN (sITLN) 1, 2 and 3 with full length sITLN 1 and 2 and 3'end of sITLN3.

2.3.15.3 Sequencing of full length sheep ITLN3

The 5' end of sITLN3 was amplified in jejunum using the forward primer sITLN F1 with the sITLN3 specific reverse primer, sITLN R9-3. The 3' end was amplified using the primers sITLN F10 and sITLN R10. The full sequence of sITLN3 was obtained from the overlapping sequences of the 5' and 3' ends and was verified in jejunum from two sheep using the full length primers sITLN F7 and sITLN R10. All primers were designed using the primer 3 programme (2.3.12). The predicted ORF, N-glycosylation sites, predicted signal peptide and GPI anchor were identified as described previously (2.3.13.6). Location of primers and details of primers used in sequencing are shown in Figure 2.5 and Table 2.5.

2.3.16 Sequencing of full length sheep SIAT4C

In September 2006 a large number of sheep expressed sequence tags (ESTs) from work undertaken in New Zealand were released to Genbank. A search of databases resulted in identification of five ESTs for sheep SIAT4C (EMBL-EE828727, EMBL-EE823877, EMBL-EE777840, EMBL-EE779830, EMBL-EE758360) which when aligned with the bovine SIAT4C sequence were shown to cover the full length sequence. Using the 5' end sequence EMBL-EE823877 (800bp), the 3'end sequence EMBL778840 (867bp) and the overlapping sequence EMBL-EE758360 (785bp) a tentative sheep sequence was identified. Using the programme primer 3, primers was designed for the 5' end (sSIAT4C F5 and R5), 3' end (sSIAT4C F6 and R6) and the full sequence (sSIAT4C F4 and R4) which were amplified from abomasal

mucosa of 2 sheep (MRI trial 2, day 5 cpi). The resultant PCR products were purified, the sequences verified, the predicted open reading frame, signal peptide, N-glycosylation sites and GPI anchor were identified as described previously (2.3.10, 2.3.11, 2.3.13.6). Location of primers and details of primers used in sequencing are shown in Figure 2.5 and Table 2.5.

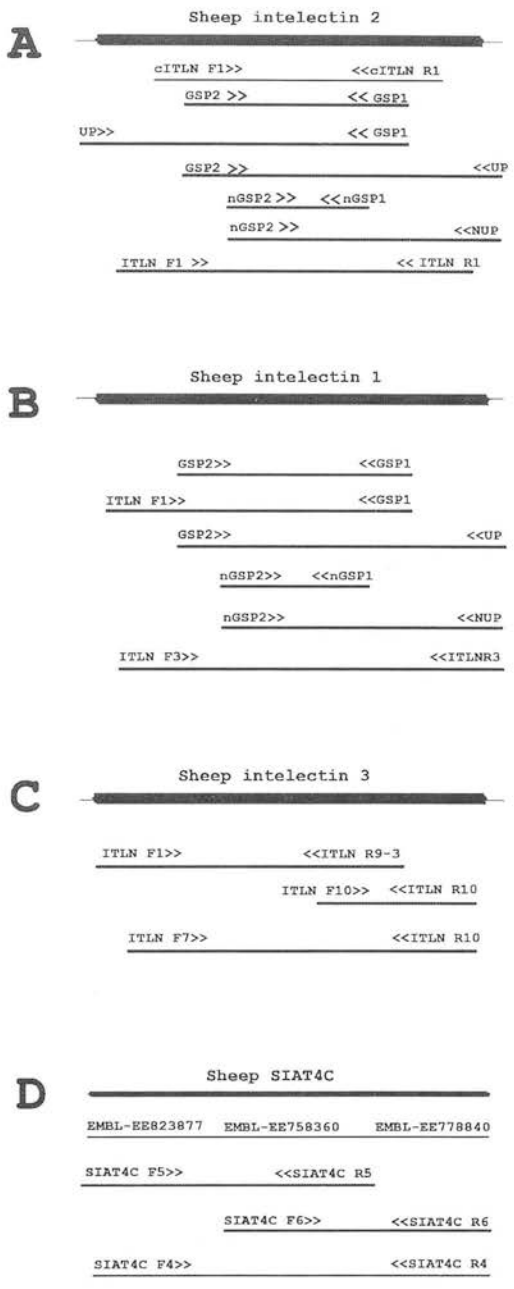


Figure 2.5: Location of primers used in sequencing sheep ITLN1 (sITLN1), sITLN2, sITLN3 and sheep SIAT4C.

The Location of primers used in the sequencing of sITLN1 (A), sITLN2 (B), sITLN3 (C) and sheep SIAT4C (D) are shown. Nested gene specific primer (nGSP), universal primer (UP).

2.3.17 Sequencing of RELMβ in sheep

RNA was extracted, reverse transcribed from sheep abomasal mucosa (MRI trial 1, group 4, day 10 sheep) and sheep respiratory tract mucosa (MRI trial 2, group 1 sheep) as described previously (2.3.1 -2.3.6). The primers, human RELMβ forward and reverse and mouse RELMβ forward and reverse primers were used to attempt to amplify a product from the sheep tissues, however no product was amplified. The conditions used for hRELMβ primers are shown in Table 2.6 and for mouse RELMβ primers the reaction conditions were as follows: 40 cycles of 94°C for 40 sec, 55°C for 40 sec and 72°C for 2 min. Sequences of primers are shown in Table 2.5. No further work was undertaken with this molecule in sheep.

2.4 Details of primers

2.4.1 Primers used for sequencing sheep ITLNs, SIAT4C and RELMβ

The details of primers used to sequence the three sheep ITLNs and sheep SIAT4C and to attempt to sequence sheep RELMβ are shown in Table 2.5.

Gene	Primer sequence		Product size
Consensus ITLN F1	Fw	CAG AAG CTG CAA GGA AAT CAA	503
Consensus ITLN R2	Rv	TTG TCA GTC CAA CAC TTT CCT	
sITLN ext GSP 2	Fw	GGT GGC GGC TGG ACC CTG GTG	309
sITLN ext GSP 1	Rv	GCT CCG GAA GAA GCC CGT GTT GG	
sITLN int GSP 2	Fw	CAC GGT GGG CGA TCG CTG GTC	176
sITLN int GSP 1	Rv	GCA CGT GCC AGA TGC CCA GGT C	
sITLN F1	Fw	GCT CTG AGA CTG CTC CTG GT	1182
sITLN R1	Rv	TTC TCC TTT GCT TGT TGG AGA	
sITLN F3	Fw	GCA TCT GTG AGG AAG GAA GG	1112
sITLN R3	Rv	TTC CAT TTC TGA GGG TCT GG	
sITLN F1	Fw	GCT CTG AGA CTG CTC CTG GT	1125
sITLN R9-3	Rv	TGG GTT CCA CAT CTC ATA GA	
sITLN F10	Fw	GCGAGAATTTGTTGCAGGAT	417
sITLN R10	Rv	CCAGGGGTCAGTCAGTTTGT	
sITLN F7	Fw	AGGGGACAGGAGTCAGGTTT	1000
sITLN R10	Rv	CCAGGGGTCAGTCAGTTTGT	
sSIAT4C F4	Fw	GATGACAACTCTCCCCAGGA	1524
sSIAT4C R4	Rv	CCCTGTGCCAGTGCTCTT	
sSIAT4C F5	Fw	AGCCGGGATGACAACTCTC	960
sSIAT4C R5	Rv	CATCATGGACTTGAGCGTGA	
sSIAT4C F6	Fw	GACCACCATGCGTCTCTTCT	863
sSIAT4C R6	Rv	CACTGTCATATGCCCCCTCT	
hRELMβ	Fw	GAA GAT CAA GGA TGT TCT CAA CAG	298
hRELMβ	Rv	TTG GGA CCC TGG TTT CAT TA	
mRELMβ	Fw	AAGGAAGCTCTCAGTCGTCAA	286
mRELMβ	Rv	GAGTCAGGTTTCCTGGTCGAG	

Table 2.5: Details of primers used in sequencing sheep genes

The forward and reverse primer sequences are shown that were used for sequencing full length sheep ITLNs 1, 2 and 3, sheep SIAT4C and to attempt to sequence sheep RELMβ. Forward (FW), reverse (RW), intelectin (ITLN), sheep (s), human (h), mouse (m)

2.4.2 Primers used for RT-PCR in human cell lines

In initial experiments beta actin was used as the housekeeping gene, however concern that it was changing with cytokine stimulation, especially at higher cytokine concentrations, led to a change to the use of ribosomal protein L19 (RPL 19). Previously published sequences for beta actin (Raff et al., 1997), RPL19 (Eisenberg and Levanon, 2003), and SIAT4C (Delmotte et al., 2002) were used. hITLN 1 primers were designed by Alan Pemberton and RELMβ primers were designed using the internet based programme PRIMER 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi). All primers were obtained from Operon, Harrogate, UK. PCR conditions for each set of primer are shown in Table 2.6.

Gene	Primer sequence		Conditions Temperature and time					Cycle No.	Product size (BP)
			ID	D	A	E	FE		
hβactin	Fw	CCT CGC CTT TGC CGA TCC	94°C 2 min	94°C 30 sec	60°C 1 min	72°C 30 sec	72°C 7 min	27	626
	Rv	GGA TCT TCA TGA GGT AGT CAG TC							
hITLN1	Fw	CAG AAG CTG CAA GGA AAT CAA	94°C 2 min	94°C 40 sec	55°C 20 sec	72°C 2 min	72°C 10 min	27	495
	Rv	TTG TCA GTC CAA CAC TTT CCT							
hSIAT4 C	Fw	CCC AAG AAC ATC CAG AGC CTC A	94°C 2 min	94°C 1 min	58°C 1 min	72°c 1 min 30 sec	72°C 7 min	27 or 32	458
	Rv	CGT GGT GGG CTT CTG CTT AAT C							
hRELM β	Fw	GAA GAT CAA GGA TGT TCT CAA CAG	94°C 2 min	94°C 1 min	58°C 1 min	72°C 1 min 30 sec	72°C 7 min	30	298
	Rv	TTG GGA CCC TGG TTT CAT TA							
hRPL19	Fw	ATCGATCGCCA CATGTATCA	94°C 2 min	94°C 1 min	55°C 1 min	72°C 1 min	72°C 5 min	25	168
	Rv	GCGTGCTTCCTT GGTCTTAG							

Table 2.6: Primers and conditions used for human cell culture RT-PCR

Forward (FW), Reverse (RW), initial denature (ID), denature (D), annealing (A), elongation (E), final elongation (FE), base pair (BP)

2.4.3 Primers and conditions used for RT-PCR in sheep tissues

Sheep ATPase was used as the housekeeping gene, primers were designed by Tom McNeilly (PHd thesis). Previously published ovine IL-4 primers (Craig et al., 2007), sheep mast cell protease 1 primers (Pemberton et al., 2000) ovine galectin 14 primers (Dunphy et al., 2002) were used. Sheep SIAT4C primers were designed from the bovine sequence using Primer 3. sITLN1, sITLN2 and sITLN3 specific primers were designed as described previously (2.3.15.2). All primers were obtained from Operon, Harrogate, UK. Final PCR conditions for each set of primer are shown in Table 2.7.

Gene	Primer sequence		Conditions Temperature and time					Cycle No.	Product size (BP)
			ID	D	A	E	FE		
sATPase	Fw	GCT GAC TTG GTC ATC TGC	94°C 2 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	72°C 7 min	31	167
	Rv	CAG GTA GGT TTG AGG GGA TAC							
sIL-4	Fw	AAC GCC GAA CAT CCT CAC AT	94°C 2 min	94°C 40 sec	55°C 40 sec	72°C 2 min	72°C 10 min	40	171
	Rv	AGT CCG CCC AGG AAT TTG TT							
sMCP-1	Fw	ACA TCG TGG ACA GAG AGA GG	94°C 2 min	94°C 30 sec	60°C 30 sec	72°C 1 min	72°C 7 min	35	354
	Rv	TCT TCC TCT TGG TTG AAT CTC							
OvGal-14	Fw	ATT CCT GTT GCA GAA GTC TAC CTG GAC A	94°C 2 min	94°C 40 sec	61°C 20 sec	72°C 2 min	72°C 7 min	34	545
	Rv	GAA CAT CTT CCA CAC GGT AGG GGT							
sSIAT4C	Fw	CTG GCA CCT GAG AAA CAT GA	94°C 2 min	94°C 40 sec	55°C 40 sec	72°C 2 min	72°C 7 min	34	207
	Rv	CGG GAG TAG TTG CCA AAG G							
sITLN9-1	Fw	GCGAGAATTTGTTGC AGGAT	94°C 2 min	94°C 40 sec	58°C 40 ec	72°C 2 min	72°C 7 min	25/ 35#	287
	Rv	TGG CTT TCT GTC TCA CAC CA							
sITLN9-2	Fw	GCG AGA ATT TGT TGC AGG AT	94°C 2 min	94°C 40 sec	58°C 40 sec	72°C 2 min	72°C 10 min	23/ 35#	286
	Rv	TGG GTT CAC ATC TCA TAG A							
sITLN9-3	Fw	GCG AGA ATT TGT TGC AGG AT	94°C 2 min	94°C 40 sec	58°C 40 sec	72°C 2 min	72°C 7 min	25/ 35#	287
	Rv	TGG GTT CCA CAT CTC ATA GA							

Table 2.7: Primers and conditions for RT-PCR in sheep tissues

Lower cycle numbers were used for transcript expression in abomasal mucosa, higher cycle numbers were used for other tissues. Forward (Fw), Reverse (Rv), initial denature (ID), denature (D), annealing (A), elongation (E), final elongation (FE), base pair (BP)

2.5 *Western blots*

2.5.1 Detection of ITLN by Western blot in cell lysates and supernatants from cultured LS174T cells

Supernatant and cell lysate from LS174T cells grown in culture medium containing recombinant hIL-13 at 1 ng/ml for 48 h were used for this experiment. Cells were lysed using 150 mM NaCl (Fisher Scientific), 10mM Tris-HCl pH 7.2 ((Sigma), 0.1% sodium dodecyl sulfate (Fisher scientific), 1% Triton X (Sigma), 1% deoxycholic acid (Sigma), 5mM EDTA (Sigma). Samples were prepared in triplicate as two different antibodies were tested.

Gels were prepared as follows: separation gel (12 % acrylamide) - consisted of 0.5 M Tris Buffer saline pH 8.8 (2.5mls), purite water (3.25 ml), Protogel (30% acrylamide, 7% bisacrylamide) (4.0 ml), 10% w/v SDS (100µl), 10% w/v ammonium persulfate (100 µl), TEMED (10 µl); stacking gel - 0.5 M Tris - HCl pH 6.8 (2.5ml), Purite water (6.1ml), Protogel (1.3ml), 10% w/v SDS (100µl), 10% w/v Ammonium Persulfate (100µl), TEMED (10µl). A reducing buffer was prepared as follows: 0.5 M Tris-HCl pH 6.8 (5ml), glycerol (4ml), 10% w/v SDS (8 ml), β-mercaptoethanol (2 ml), 0.1% w/v bromophenol blue (1 ml). Samples were diluted 50:50 with reducing buffer, heated in a boiling water bath for 3 min, loaded onto gels and electrophoresed (Mini-protean -11, Bio-Rad) at 200V for 37 min.

Gels were blotted (Transblot SD, Bio-Rad) onto polyvinylidene flouride (PVDF) membrane (Immobilon P, Millipore), 160 mA for one hour (80mA/gel). The MW marker lanes were cut off and stained with Coomassie blue and then placed in destain. Blocking buffer was prepared as follows; 50 mM Tris-HCl pH 7.5, 0.15M NaCl (Sigma), 1% milk powder (Marvel), 0.5% Tween 80. Membranes were placed in blocking buffer on the shaker for one hour to block non specific protein binding, and then probed for one hour with the two different affinity purified chicken polyclonal antibodies to ITLN peptides 1 and 2, corresponding to residues 130-143 of mITLN1b/2 and residues 302-313 of mouse ITLN1b/2 respectively, and control chicken IgY, all at a concentration of 1 ug/ml. Following rinsing with ELISA buffer,

membranes were incubated for one hour with the secondary antibody, rabbit anti-chicken IgY (Sigma), at a dilution of 1:10,000. After a further rinse with ELISA buffer, membranes were dipped in purite water before staining with 3,3'-diaminobenzidine (DAB kit Vector laboratories) until bands were visualised. Membranes were dried on filter paper and aligned with MW markers.

2.5.2 Detection of ITLN and beta actin protein by Western blot in sheep abomasal tissue

Abomasal tissue (50mg) was homogenised using a Stratech Beadbeater-B (Stratech) with $1mm^3$ Zirconia/silica beads (Thistle Scientific). A urea buffer was used for protein extraction: 8 M urea ultra (Sigma), 2% CHAPS (Sigma), 0.4% dithiothetol (Sigma) and protease inhibitors (complete mini, EDTA-free, Roche). 50 µg of protein was run on 10% acrylamide SDS-PAGE mini gels (Criterion, Bio-Rad, California, USA). Equal loading of proteins was checked by staining gels with Imperial stain (Pierce, Rockfort, USA) for 1 h before rinsing and visualising with a transilluminator. Gels were blotted (Transblot SD, Bio-Rad) onto polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore) and blocked with 50 mM Tris-HCl pH 7.5, 0.15M NaCl, 2% milk powder, 0.5% Tween 80. Blots were incubated overnight at 4°C with both affinity purified chicken polyclonal antibody to ITLN peptide (French et al., 2007) (0.45 µg/ml) and also mouse antibody to beta-actin (Sigma Clone, AC 15; 1:10,000) in blocking buffer. Following washing, membranes were incubated for 1 h with goat anti-mouse alkaline phosphatase antibody 1:20,000 (Jackson ImmunoResearch), washed again and incubated for a further 1 h with the horseradish peroxidase conjugated rabbit anti-chicken IgY (Sigma, A9046), at a dilution of 1:10,000. ITLN specific labelling was visualised using chemilumiscent peroxidase substrate (Sigma) and after washing beta actin specific labelling was visualised using CPD – Star substrate (Boehringer-Mannheim, Germany). Images were acquired using the Kodak Digital Science ImageStation 440CF. This procedure allowed both the protein of interest (ITLN) and a housekeeping protein (beta actin) to be visualised on the same blot.

2.6 Immunohistochemistry, immunofluorescence and carbohydrate histochemistry

2.6.1 Optimisation of ITLN immunohistochemistry for sheep tissues

Sections collected from trachea and lung of normal sheep (MRI trial 2, group 1) were fixed in 4% paraformaldehyde and submitted to the Division of Veterinary Clinical Pathology at the R(D)SVS for paraffin embedding and cutting of 4 μm thickness serial sections onto coated slides. Sections were dewaxed in xylene for 10 min, rehydrated through graded alcohols and water. Endogenous peroxide activity was blocked by immersing in 3% hydrogen peroxide in methanol for 20 min.

All sections were stained with three different anti-ITLN antibodies for comparison: affinity purified antibodies to ITLN peptide 1 and ITLN peptide 2 and a rabbit polyclonal antibody against frog egg lectin XL35 IgG (Lee et al., 2000). The imunohistochemistry protocol and concentration was optimised for each antibody. For anti-ITLN Peptides 1 and 2 antigen retrieval produced better results, and 1mM EDTA pH 8, Tris-HCL pH 9 or Tris EDTA with 0.05% Tween 80 appeared superior to 10 mM citric acid pH 6. For the anti-XL35 antibody antigen retrieval produced negative results and was not included in the protocol. With all antibodies staining appeared less consistent when a Shandon Sequenza (Thermo Fisher Scientific, Surrey, UK) was used and thus slides were stained by lying on plastic pipettes in a tray lined with moist paper towel. A wax pencil was used to delineate the area for staining. For antigen retrieval sections were placed in buffer and microwaved (Panasonic 800W D) in a pressure cooker (Biomen pressure cooker, A Menarini Diagnostics) that had reached full pressure, for 4 min. Slides were washed, blocked with PBS containing 10% normal rabbit serum 0.5 M NaCl and 0.5% Tween 80, and incubated with affinity-purified chicken anti-ITLN peptide 1 or 2 (1, 2 or 5 μg/ml in blocking buffer) or control chicken IgY (1, 2 or 5 μg/ml in blocking buffer) for 1 h at 21°C. After treatment with horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgY (Sigma, 1/500), sections were stained with Nova Red (Vector Laboratories), counterstained with hematoxylin, then dehydrated and mounted. For

anti-XL35 the antigen retrieval step was omitted, slides were blocked with PBS containing 0.5 M NaCl and 0.5% Tween 80, and incubated with anti-XL35 Ig or control rabbit IgG for 1 h at 21°C. After treatment with biotinylated donkey anti-rabbit IgG (BA4001, 1/400; Vector Laboratories, Burlingame, CA), followed by avidin-HRP conjugate (ABC kit; Vector Laboratories) sections were stained with diaminobenzidine (DAB kit Vector laboratories), counterstained with hematoxylin, then dehydrated and mounted. Sections were examined using a Leitz light microscope (Wetzlar, Germany).

2.6.2 Final protocol for ITLN immunohistochemistry of sheep tissues

The affinity purified antibody to ITLN peptide 1 (5 μg/ml) was selected as the antibody of choice for use on sheep abomasum (MRI trial 1 and 2), for sheep tracheal explants, sheep normal respiratory tissue (MRI trial 2, group 1) and multiple normal sheep tissues (MRI trial 3, group 1). An antigen retrieval step was included in the protocol using Tris-HCl EDTA pH 9 with 0.05% Tween 80 as the antigen retrieval buffer. For full details of the protocol see 2.6.1.

2.6.3 Immunofluoresence of LS174T cells to examine structural change after incubation with recombinant human interleukin 4

Sytox green (1:20,000) dilution and phalloidin (5 units in 1 ml) were prepared in PBS/Tween 80. Coverslips with LS174T cells (2.1.1) that had been incubated for 48 h in medium only or medium plus recombinant human IL-4 at 10 ng/ml were incubated with either phalloidin, sytox green or a combination of both for 20 min in the dark. Coverslips were washed twice with PBS pH 7.4, carefully lifted from chambers and mounted on slides with Mowiol. They were left for 24 h in the dark before examining and capturing images with an MRC-600 confocal laser scanning microscope (CLSM: Bio-ad Laboratories) mounted on an Axiovert 10 inverted microscope equipped with Plan-Apochromat objective lenses (Carl Zeiss).

2.6.4 Carbohydrate histochemistry - Alcian blue/ Periodic acid Schiff staining

Sections fixed in 4% paraformaldehyde were dewaxed in xylene, rehydrated by passing through graded alcohols and water and incubated for 5 min with 1% Alcian blue pH 2.5 to show acidic mucus. After rinsing in distilled water sections were incubated in 1% periodic acid for 10 min and rinsed again before immersion in Schiff's reagent for 20 min to demonstrate neutral mucus. Sections were washed in running tap water for 10 min, counterstained with Mayer's haemotoxylin, blued with Scott's tap water, dehydrated and mounted with DPX mountant (Sigma).

2.7 Enumeration of cells

2.7.1 Enumeration of ITLN positive cells in sheep tracheal explants

Because of the sparsity of positively staining goblet cells, positive cells were enumerated by counting 5 fields at a resolution of x 25 and the results for triplicate sections were added together rather than using a mean.

2.7.2 Enumeration of goblet cells in tracheal explants

The goblet cells were enumerated by counting 5 fields at x 40 resolution in triplicate sections using a Leitz light Microscope (Wetzlar, Germany).

2.8 Statistical analysis

A two-tailed unpaired or paired student t-test was used for comparison of two groups when data was normally distributed. Normality was determined using the Kolmogorov Smirnov test. If data was not normally distributed the Mann-whitney U test was used. When multiple groups were compared the Kruskal-Wallis test was used to determine if there was significant difference between groups and a Mann-whitney U test was used as a post hoc test for all pairwise comparisons; p-values < 0.05 were considered significant. Spearman's rank correlation coefficient was used

to determine correlation between sets of data. All analysis was carried out using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

3 *In vitro* studies of SIAT4C, RELMβ and ITLN gene expression

3.1 Summary

The effect of cytokines on the expression of SIAT4C, RELMβ and ITLN transcripts was examined using *in vitro* cell culture. Two human mucoid cell culture lines were chosen, LS174T and NCI-H292. In the LS174T cell line there was no significant change in expression of SIAT4C transcript following culture with IL-9, IFNγ or TNFα at 1ng/ml for 48 hours or either IL-4 or IL-13 for 12 or 48 hours at 1ng/ml or 10ng/ml compared to controls. However using this same cell line there was significant increase in expression of RELMβ following incubation with IL-4 at 1 or 10 ng/ml for 48 hours and ITLN was significantly increased following incubation with either IL-4 or IL-13 (1 or 10ng/ml) at 12 and 48 hours compared to controls. In the NCI-H292 cell line whilst there was no significant change in expression of SIAT4C transcript following culture with IL-4, IL-13, IL-9 or TNFα at 1ng/ml for 48 hours there was significant decrease in expression following culture with IFNγ at the same concentration. The house keeping gene beta actin was used initially, but because of a trend to upregulation following culture with the cytokine IL-13, was replaced with the ribosomal gene RPL19. Incubation of LS174T cells with either of the Th2 cytokines IL-4 or IL-13 resulted in cell morphological changes which were studied further using confocal microscopy.

3.2 Introduction

Upregulation of SIAT4C (Knight et al., 2004), RELMβ (Artis et al., 2004; Knight et al., 2004) and ITLN1b/2 (Pemberton et al., 2004a; Artis, 2006; Voehringer et al., 2007) transcripts, all mucus related molecules, has been shown in nematode infections in mice responding with a typical Th2 response. *In vitro* studies have shown upregulation of RELMβ transcript in response to culture with the Th2

cytokines, IL-4 and IL-13 (10ng/ml), and downregulation with the Th1 cytokine IFNγ using the human colonic mucoid adenocarcinoma LS174T cell line (Artis et al., 2004). Using this same cell line Julie Bethune (MSc thesis, University of Edinburgh, 2005) has shown upregulation of hITLN transcript in response to culture for 48 hours with the Th2 cytokines, IL-4 and IL-13 (1ng/ml) whilst no change was seen with IL-1β, IL-9, IFNγ or TNFα at the same concentration. Whilst there are reports in the literature of upregulation of SIAT4C transcript in a human respiratory glandular cell line, MM-39, and in human bronchial mucosa explants following incubation with TNFα at 20ng/ml for 18 hours there arre no reports of regulation of SIAT4C by Th2 cytokines (Delmotte et al., 2001; Delmotte et al., 2002). The objective of this experiment was to determine if there is regulation of SIAT4C transcript by selected Th1 and Th2 cytokines in the gastro-intestinal tract and/or respiratory tract using *in vitro* cell culture and furthermore to see if there is co-regulation with RELMβ and ITLN in response to incubation with the Th2 cytokines IL-4 and IL-13. The human colonic mucoid adenocarcinoma cell line, LS174T and the human mucoepidermoid carcinoma respiratory cell line, NCI-H292, were used to determine the expression of SIAT4C transcript in response to incubation with selected Th1 and Th2 cytokines and the LS174T cell line was used to examine co-regulation of the three genes of interest in response to selected Th2 cytokines. The materials and methods used are described in detail in chapter 2. A Kruskal –Wallis test was used to compare groups and a Mann-Whitney U test was used a post hoc test for all pairwise comparisons.

3.3 Results

3.3.1 Expression of SIAT4C in response to cytokines

The expression of SIAT4C in response to IL-4, IL-13, IL-9, IFNγ and TNFα in NCI-H292 cells and LS174T cells was determined. NCI-H292 cells (4 replicates) were grown in medium alone or medium containing either recombinant human IL-4, IL-9, IL-13, IFNγ or TNFα at 1 ng/ml for 48 h (2.2.1). The cells grown in medium containing IL-4 or IL-13 appeared to grow faster. RNA was isolated, reverse transcribed, and semi-quantitative RT-PCR carried out following optimisation of thermocycle number to amplification stage of the genes of interest as described

previously (2.3.1.1, 2.3.2 – 2.3.8, 2.4.2, Table 2.6). Gene expression was normalised to the house keeping gene beta actin which on RT-PCR had shown consistent expression in all cells. SIAT4C showed strong constitutive expression in cells grown in medium alone. A Kruskal-Wallis test was used to compare transcript expression between groups (p = 0.1080). As no significant difference was found between groups, no post hoc tests were applied (Figure 3.1A).

LS174T cells (4 replicates) were grown in medium alone or medium containing either recombinant human IL-4, IL-9, IL-13, INFγ or TNFα at 1 ng/ml for 48 h (2.2.1). As seen with the NCI-H292 cell line, cells grown in medium containing either IL-4 or IL-13 appeared to grow faster, furthermore the LS174T cells also showed morphological changes within 24 h with clustering of cells occurring. RNA was isolated, reverse transcribed, and semi-quantitative RT-PCR carried out following optimisation of thermocycle number to amplification stage of the genes of interest as described previously (2.3.1.1, 2.3.2 – 2.3.8, 2.4.2, Table 2.6).

On RT-PCR, strong constitutive expression of SIAT4C was shown in this cell line. Beta actin was used as a house keeping gene and on RT-PCR transcript expression was not as consistent as had been seen in the NCI-H292 cell line, and there was concern that there was upregulation of beta actin in the cells grown in medium containing IL-13. The results were analysed nevertheless. A Kruskal-Wallis test demonstrated no significant difference in transcript expression between groups (p = 0.0785). As no significant difference was found between groups, no post hoc tests were applied (Figure 3.1B).

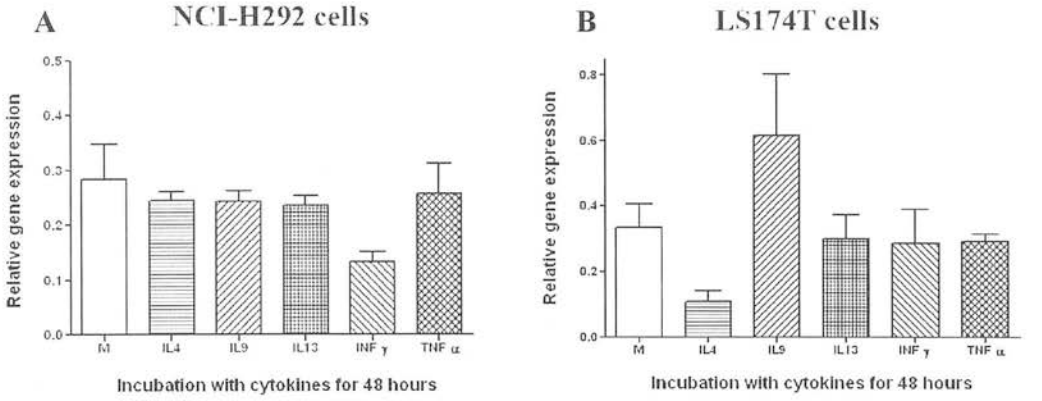


Figure 3.1: Effect of cytokines on expression of SIAT4C transcript

Semi-quantitative RT-PCR showing relative expression of SIAT4C transcript in NCI-H292 cells (A) and LS174T cells (B) following incubation in medium alone (M) or medium with recombinant human IL-4, IL-9, IL-13, IFNγ or TNFα at 1ng/ml for 48 hours. Expression is relative to the housekeeping gene beta actin. For all cell cultures n = 4. Bars represent mean + SEM.

3.3.2 Expression of ITLN, RELMβ and SIAT4C in response to Th2 cytokines

The expression of ITLN, RELMβ and SIAT4C in response to recombinant human IL-4 or IL-13, at different time points, and at different concentrations of cytokines in LS174T cells was determined. LS174T cells (4 replicates) were grown in medium alone or in medium containing recombinant human IL-4 or IL-13 (1 ng/ml or 10 ng/ml) (2.2.1). Cells were harvested at 12 h and 48 h. RNA was isolated, reverse transcribed, and semi-quantitative RT-PCR carried out following optimisation of thermocycle number to amplification stage of the genes of interest as described previously (2.3.1.1, 2.3.2 – 2.3.8, 2.4.1, Table 2.6). Gene expression was normalised to the house keeping gene RPL-19. A Kruskal-Wallis test was used to compare all groups and as a significant difference was found between groups, a Mann-Whitney U test was applied as a post hoc test for pairwise comparisons (Figure 3.2).

ITLN transcript expression was not present in cells grown in medium alone and was induced at 12 h and at 48 h in cells incubated with either IL-4 or IL-13 at 1 ng/ml or 10 ng/ml. The higher concentration of IL-13 (10 ng/ml) at 48 h resulted in a significantly greater expression of ITLN compared to cells grown in IL-13 at 1 ng/ml ($p = 0.0286$).

RELMβ was constitutively expressed and interestingly there was downregulation when incubated with medium containing IL-4 for 12 h at 1 ng/ml ($p = 0.0571$) which reached significance at 10ng/ml ($p = 0.0286$) and significant upregulation following incubation with IL-4 at either 1 ng/ml ($p = 0.0286$) or 10ng/ml ($p = 0.0286$) for 48 h when compared to controls. There was a trend to increased expression following incubation with IL-13 at 10 ng/ml ($p = 0.0571$) at 48 h however this failed to reach significance and no significant change in expression was seen following incubation with IL-13 for 48 h at 1ng/ml ($p = 0.3429$) or for 12 hours at 1 ng/ml ($p = 0.3429$) or 10 ng/ml ($p = 0.2$) compared to controls.

Expression of SIAT4C remained unaltered following incubation for 12 h with IL-4 at 1ng/ml ($p = 0.6857$) or 10 ng/ml ($p = 0.1143$) and for 48 h at 1 ng/ml ($p = 0.3429$) or 10 ng/ml ($p = 0.1143$). Likewise, no significant change in expression was seen following incubation with IL-13 at 1ng/ml for 12 h ($p = 0.6857$) or 48 h ($p = 0.2$), or with IL-13 at 10 ng/ml for 12 h ($p = 0.6857$) or 48 h ($p = 0.4857$).

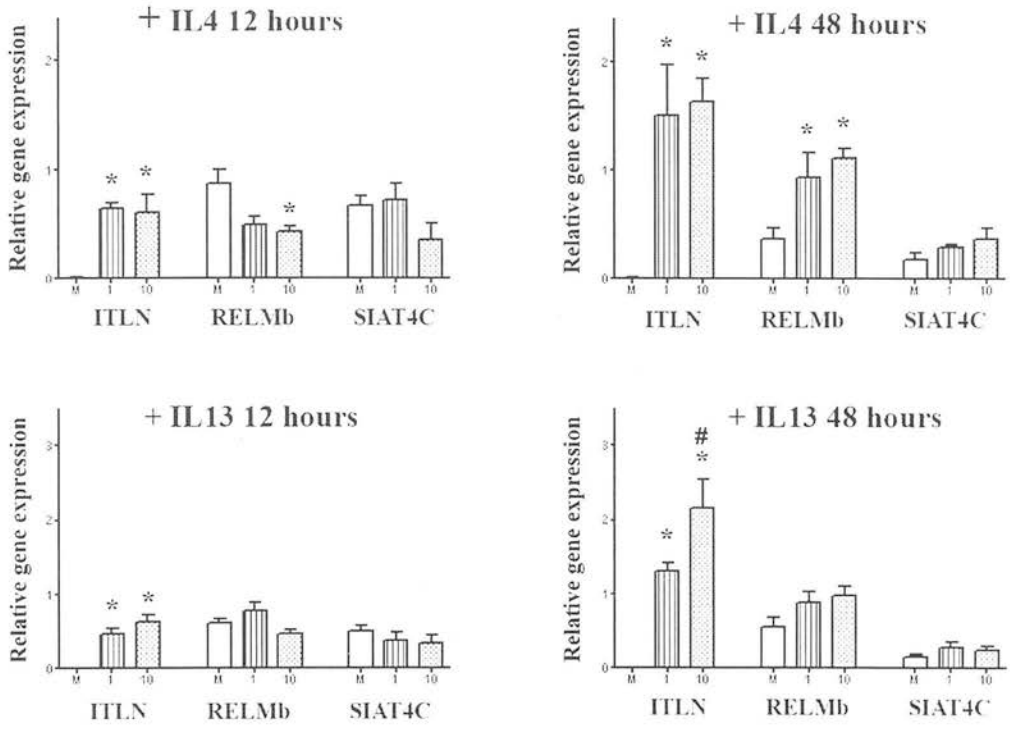


Figure 3.2: Effect of Th2 cytokines on expression of ITLN, RELMβ and SIAT4C transcripts

Semi-quantitative RT-PCR showing relative expression of ITLN, RELMβ and SIAT4C transcripts in LS174T cells incubated with medium alone (M) or with medium containing IL-4 or IL-13 at 1ng/ml (1) or 10ng/ml (10) for 12 or 48 hours. Expression is relative to the housekeeping gene RPL-19. For all cell cultures n = 4. Bars represent mean + SEM. * Significant difference compared to medium only, # significant difference between 10 ng/ml and 1 ng/ml.

3.3.3 Expression of beta actin in response to Th2 cytokines

The expression of beta actin transcript in response to IL-4 or IL-13 at different time points and at different concentrations of cytokines in LS174T cells was determined. In experiment 1 there was concern that the transcript of the house keeping gene beta actin may have upregulated in LS174T cells in response to incubation with IL-13 for 48 h compared to controls. In order to further investigate this observation the expression of beta actin transcript was examined in LS174T cells (four replicates)

grown in IL-4 (1 ng/ml and 10 ng/ml) and IL-13 (1 ng/ml and 10 ng/ml) for 12 and 48 h. RNA was isolated, reverse transcribed, and semi-quantitative RT-PCR carried out as described previously (2.3.1.1, 2.3.2 – 2.3.8, 2.4.2, Table 2.6). Beta actin transcript expression was normalised to the house keeping gene RPL19 which had been shown to be consistent in expression in experiment 2.

A Kruskal-Wallis test was used to compare all groups and as no significant difference was found between groups no post hoc test for pairwise comparisons was applied. Whilst no significant difference was noted in beta actin transcript following incubation with IL-13 compared to controls it was noted that expression was less consistent and showed more variation than had been noted following incubation of this cell line with IL-4 (Figure 3.3).

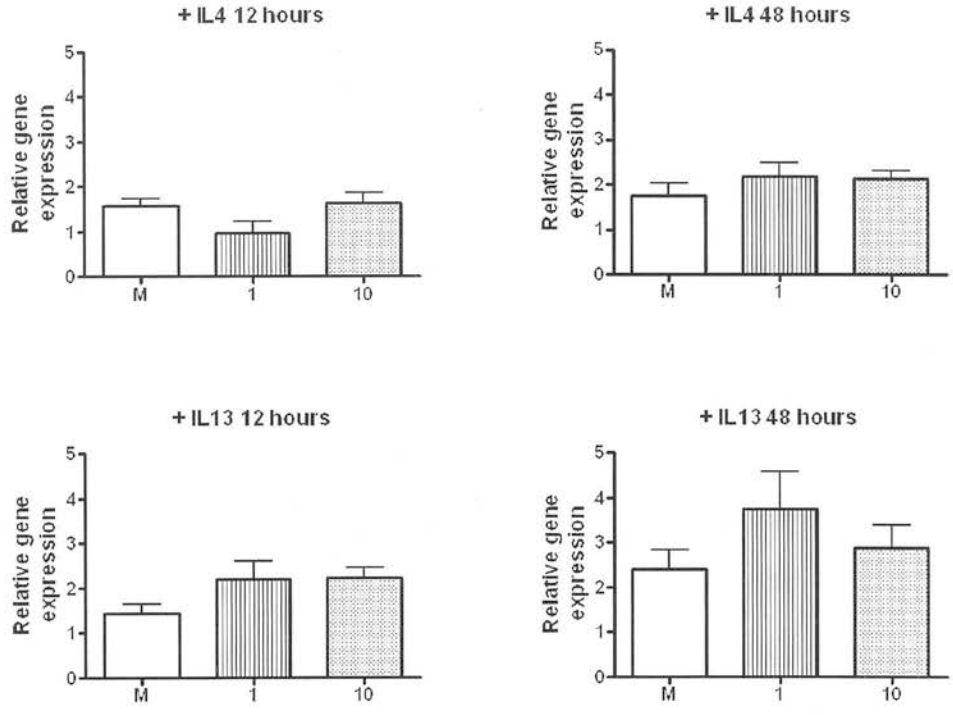


Figure 3.3: Effect of Th2 cytokines on expression of beta actin

The expression of Beta actin transcript in LS174T cells in medium alone and when incubated with IL-4 or IL-13 at 1ng/ml (1) and 10ng/ml (10) for 12 and 48 hours. Expression is relative to the house keeping gene RPL19. There was no significant change in expression of Beta actin following incubation with either IL-4 or IL-13;

however expression was shown to be less consistent with IL-13 compared to IL-4. For all cultures n = 4. Bars represent mean + SEM.

3.3.4 Immunolabelling of LS174T cells

In order to further examine the observed change in morphology of LS174T cells when incubated with either IL-4 or IL-13, LS174T that had been cultured on cover slips for 48 h with medium alone or medium containing recombinant IL-4 at 10 ng/ml were immunolabelled with Sytox green and phalloidin as described in chapter 2 (2.2.2, 2.6.3). Sytox green specifically stains nucleic acid whilst phalloidin stains filamentous actin. Images were captured with an MRC-600 confocal laser scanning microscope (CLSM: Bio-Rad Laboratories) mounted on an Axiovert 100 inverted microsope equipped with Plan-Apochromat objective lenses (Carl Zeiss). Cells grown in medium alone appeared to spread out on the coverslip whilst cells grown in medium plus IL-4 tended to form clumps. The phalloidin staining in cells grown in IL-4 appeared more intense in areas which might suggest a change in expression of filamentous actin (Figure 3.4).

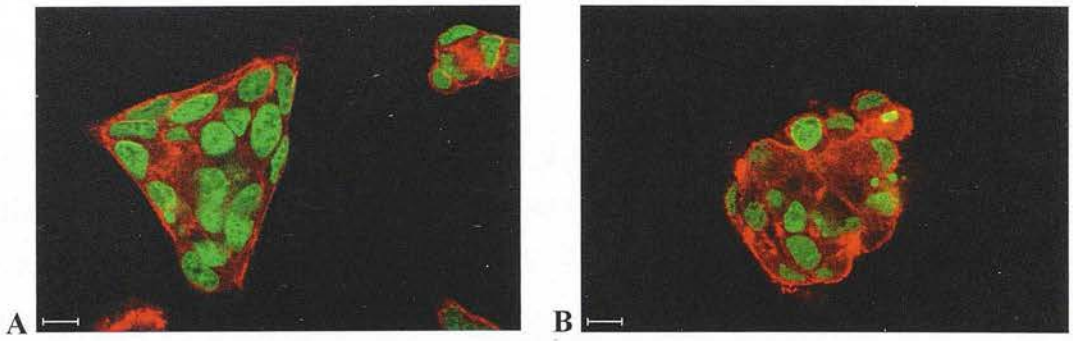


Figure 3.4: LS174T cells grown on glass coverslips in (A) medium or (B) medium + IL-4 (10 ng/ml) and stained with phalloidin/sytox green. Note the clumping of the cells grown in IL-4. Also note the increased intensity of the phalloidin stain in B.

3.4 Discussion

3.4.1 Effects of cytokines on expression of SIAT4C transcript

SIAT4C has been shown to be expressed in a wide variety of human tissues (Kitagawa and Paulson, 1994a) and was constitutively expressed in the two human cell lines studied. Previous studies have shown that SIAT4C transcript is upregulated in human bronchial explants and the human respiratory glandular cell line, MM-39, when stimulated with the proinflammatory cytokine TNFα (Delmotte et al., 2001) and the human hepatic carcinoma cell line HuH-7 following incubation with IL1β (Higai et al., 2006). In the present study the cytokines IL-4, IL-9, IL-13, IFNγ and TNFα did not significantly alter transcript expression at the time points and concentration of cytokines used in either cell line. Interestingly in previous studies a higher concentration of cytokine (TNFα, 20ng/ml; IL1β, 100 u/ml) was used (Delmotte et al., 2001; Higai et al., 2006). The effect of different cytokine concentration is seen clearly in Figure 3.2 where 10ng/ml of IL-13 resulted in significantly higher expression of ITLN compared to 1ng/ml at 48 h. It is possible that a higher concentration of cytokine may have resulted in different results in these experiments.

Altered expression of SIAT4C has been shown in carcinomas (Saito et al., 2002; Kemmner et al., 2003; Wang et al., 2005) and it is possible that use of cell lines from normal tissue would have been preferable. Further work is required to validate the present finding and to determine the significance.

3.4.2 Effect of Th2 cytokines on expression of RELMβ transcript

RELMβ is a mucus associated molecule and previous studies have shown transcript to be upregulated in LS174T cells by incubation with the Th2 cytokines, IL-13 or IL-4 at 10ng/ml (He et al., 2003; Artis et al., 2004) and decreased by incubation with IFNγ. The upregulation of RELMβ has been found to be IL-4Rα dependent and most likely STAT6 induced (Artis et al., 2004). The results of the present experiment are in agreement with previous studies, with significant upregulation of RELMβ transcript seen following incubation with IL-4 (10ng/ml) for 48 h, however this failed to reach significance for incubation with IL-13. The apparent downregulation of RELMβ transcript following culture with IL-4 (1 ng/ml) for 12 h may have been artefactual as expression of RELMβ transcript in the medium only controls was not consistent, showing some variation between experiments. The effect of proinflammatory cytokines on the expression of RELMβ were not determined in this study, however it is of particular interest that recent work using the LS174T cell line has demonstrated the ability of TNFα (100 ng/ml) and insulin to upregulate RELMβ transcript expression (Fujio et al., 2007). The presence of a nuclear factor B binding site in the RELM promoter has led to speculation that TNFα induced NF-B activation may be a critical pathway in optimal effector response of RELMβ in the gastro-intestinal tract (Artis et al., 2004). Interestingly RELMβ may also stimulate naïve bone marrow derived macrophages to secrete TNFα, IL-6 and RANTES (regulated upon activation, normal T cell expressed and secreted), support for a role for RELMβ in inflammation (Barnes et al., 2007).

3.4.3 Effect of Th2 cytokines on expression of ITLN transcript

ITLN was shown to be induced by both IL-4 and IL-13 at 12 h with a further increase in expression shown at 48 h. This result is in agreement with previous findings (Julie Bethune, MSc thesis, University of Edinburgh 2005) and furthermore demonstrates the very early induction of ITLN by these cytokines. Interestingly ITLN was found to be the most upregulated gene in a microarray analysis of this

human mucoid colonic cell line following incubation with IL-4 at 1ng/ml for 48 hours (Julie Bethune, MSc thesis, University of Edinburgh 2005). Previous work has shown the upregulation of ITLN in typical Th2 responses in the respiratory tract of man (Kuperman et al., 2005) and the gastrointestinal tract of mice (Pemberton et al., 2004a; Artis, 2006) and the present work confirms the regulation of this molecule by Th2 cytokines. More recent work has confirmed that this regulation is STAT 6 dependent (Voehringer et al., 2007). Whilst the function of ITLN is uncertain, the increased expression of mITLN1b/2 at the time of expulsion of *T. spiralis* in resistant mouse strains and the apparent absence of mITLN1b/2 in the genome of mouse strains which show slower expulsion of worms, would suggest a protective role against parasites. Whilst the present work supports the induction of ITLN in Th2 type response, it is of interest that recent work in fish would suggest that this may not be the only mechanism of control of ITLN with upregulation seen in response to acute bacterial inflammation (Gerwick et al., 2007).

3.4.4 Morphological changes of cells and rate of growth of cells

The reason for the morphological changes seen in the LS174T cell following incubation with IL-4 or IL-13 was not ascertained. It is possible that the induction of ITLN may have a role to play. Previous work has shown that ITLN may alter the jelly coat in frog eggs to prevent polyspermy (Nishihara et al., 1986). Further studies using ITLN gene inhibitors may help further characterise the role of ITLN in this morphological change.

The apparent increased rate of growth of both cell lines when cultured with either IL-4 or IL-13 was an interesting observation. Previous studies have shown proliferation of epithelial cell in response to IL-4 (McGee and Vitkus, 1996) and IL-13 (Booth et al., 2001). A proliferation assay would have confirmed this subjective observation.

3.4.5 Co-regulation of ITLN, RELMβ and SIAT4C

Whilst in the model of *T. spiralis* infection in mice (Knight et al., 2004; Pemberton et al., 2004a), a known inducer of a Th2 response, ITLN, RELMβ and SIAT4C were all shown to upregulate, the studies presented in this chapter have shown regulation of RELMβ and ITLN by Th2 cytokines but have failed to show co-regulation with SIAT4C. The experiments highlighted the influence of cytokine concentration and the importance of cell line selection. It is possible that further studies with higher concentration of cytokines and with different cell lines may have resulted in different results.

3.5 Conclusion

The *in vitro* results presented in this chapter suggest that the mucus associated molecules of interest, ITLN, RELMβ and SIAT4C are not co-regulated by Th2 cytokines in the human cell lines studied. It is unknown if the same applies in a large animal model. As a result of these experiments it was decided to focus studies on the expression of the three molecules, in the gastrointestinal and respiratory tract of sheep using *in vitro*, *ex vivo* and *in vivo* methods. Sheep were chosen because of the economic importance of gastro-intestinal parasitic infections in ruminants (Claerebout et al., 2003; Coles et al., 2006) and also because of their role as models for allergic respiratory disease in man (Abraham et al., 2005; Snibson et al., 2005; Koumoundouros et al., 2006; Kasaian et al., 2007).

4 Sheep ITLN, SIAT4C and RELMβ – sequences and tissue expression

4.1 Summary

The full sequences of three sheep ITLNs and one sheep SIAT4C were determined and transcript tissue distribution established. The deduced amino acid sequences of the three sheep ITLNs shared 85 – 91% homology. Expression of sITLN2 transcript was restricted to the abomasum in normal sheep whilst sITLN1 and sITLN3 had a wider tissue distribution. SIAT4C transcript was expressed in all tissues examined. There was no evidence of RELMβ transcript expression in sheep tissues examined.

4.2 Introduction

Sheep have been shown to be an excellent model for the study of human respiratory tract disease and in particular asthma, a Th2 mediated disease (Abraham et al., 1999; Bischof et al., 2003; Abraham et al., 2005; Snibson et al., 2005). Gastro-intestinal parasites are of considerable economic importance in sheep (Claerebout et al., 2003; Newton and Meeusen, 2003) and the problem has increased in recent years due to the emergence of resistance to many of the common anthelminthics (Wolstenholme et al., 2004; Wrigley et al., 2006). Sheep nematodes have been shown to elicit a Th2 biased response (Hein et al., 2004; Pernthaner et al., 2006; Craig et al., 2007). It is for this reason it was decided to use sheep as a model to further study the co-regulation of the mucus associated molecules ITLN, RELMβ and SIAT4C which have been shown to be upregulated in a Th2 biased nematode infection in mice ((Knight et al., 2004; Pemberton et al., 2004a).

Previous work demonstrated expression of ITLN transcript and protein in the respiratory tract of sheep (Julie Bethune, MSc thesis, University of Edinburgh 2005) however it was unknown if expression occurred in the gastrointestinal tract.

Furthermore two ITLN homologues with different distribution patterns had been described in man (Lee et al., 2001; Suzuki et al., 2001; Tsuji et al., 2001) and in mice (Komiya et al., 1998; Pemberton et al., 2004a) and it was unknown if more than one ITLN was expressed in sheep. Whilst expression of SIAT4C transcript had been described in several species in a wide range of tissues there was no publication to suggest that SIAT4C was expressed in sheep. Finally, expression of RELMβ transcript and protein had been shown in the gastro-intestinal tract of mice and its human homologue FIZZ2 has been described in the gastrointestinal tract of man, however there were no publications to suggest that a homologue was expressed in sheep.

The object of this work was to determine if these molecules were expressed in sheep and if present to determine tissue distribution. All details of materials and methods used have been described previously (2.3.1 – 2.3.6, 2.3.10 – 2.3.15, Table 2.5, Figure 2.5). sITLN2 was sequenced first followed by sITLN1 and then sITLN3. However results are presented here in numerical order with sITLN1 first..

4.3 Results

4.3.1 Cloning and sequencing of sheep ITLN 1

A 220 bp partial sequence of sITLN1 was amplified from lungs of six normal sheep (MRI trial 2, group 1 sheep). Sequences in all sheep were similar and found to share 94% homology with the sITLN2 partial sequence obtained from *T. circumcincta* infected sheep abomasum (see 4.3.2) and 98% homology with the sheep respiratory tract ITLN partial sequence (GenBank accession number AM087961). The full sequence of sITLN1 was then cloned and sequenced as described in Materials and Methods (2.3.14) and was confirmed in lung samples from two sheep and tracheal mucosal samples from two sheep (MRI trial 2, group 1) using the primers sITLN F3 and sITLN R3. Using web based programmes the predicted open reading frame was identified, homology ascertained, and predicted signal peptides and N-glycosylation sites determined as described previously (2.3.13.6). The full sequence (1226 bp) thus obtained had a predicted opening reading frame of 969 bp and deduced amino

acid sequence, 322 AAs (GenBank accession number AM087961, updated October 2007). The full sequence shares 77% homology with hITLN1 (GenBank accession number NM017625) and 88% and 86% homology with sITLN2 and 3 respectively (see 4.3.2 and 4.3.3). The deduced amino acid sequence of sITLN1 shares 80% homology with hITLN1 and 86% and 85% homology with sITLN2 and 3 respectively. A 24 AA predicted signal peptide (probability 1.0) with a predicted cleavage site (probability 0.883) between AA 24 and 25 (GRG-AV) was identified, and a predicted N-glycosylation site was identified at AA 72 (Figure 4.1A). There was no evidence of a GPI anchor site. Cysteine residues implicated in oligomer formation are shown in Figure 4.1B.

4.3.2 Cloning and sequencing of sheep ITLN2

The consensus primers, cITLN F1 and cITLN R2, based on known human and mouse ITLN sequences, were used to amplify a partial sequence of sITLN2 from *T. circumcincta* infected sheep abomasum. This partial sequence shared 90% homology with hITLN1 (GenBank accession number NM017625) and 94% homology with the sheep respiratory tract ITLN partial sequence (GenBank accession number AM087961). The full abomasal sequence was then cloned and sequenced as described in materials and methods (2.3.13). Using primers sITLN F1 and R1 the full sequence was confirmed in abomasal mucosal samples of five *T. circumcincta* infected sheep (MRI trial 1, group 4, day 10) and was found to be identical. Using web based programmes the predicted open reading frame was identified, homology ascertained, and predicted signal peptides and N-glycosylation sites determined as described previously (2.3.13.6). The full sequence (1232 bp) thus obtained had a predicted opening reading frame of 972 bp and deduced amino acid sequence, 323 AAs (GenBank accession number EF521881). The full sequence shares 76% homology with hITLN1 (GenBank accession number NM017625), 88% homology with sITLN1 and 91% homology with sITLN3. The deduced amino acid sequence of sITLN2 shares 79% homology with hITLN1, 86% homology with sITLN1 and 91% homology with sITLN3. A 25 AA predicted signal peptide was identified (probability 0.971), with a predicted cleavage site (probability 0.255) between AA 25

and 26 (PRA-AG). Two predicted N-glycosylation sites were identified at AA 173 and 286 in contrast to one in sITLN1 (Figure 4.1A). A GPI anchor was predicted using the programme GPI-SOM. Cysteine residues implicated in oligomer formation are shown in Figure 4.1B.

4.3.3 Cloning and sequencing of sheep ITLN3

The 3'end of sITLN3 was obtained from the VTRI cDNA library, clone 9264. The 5' end of sITLN3 was amplified by using the forward primer sITLN F1, which had been used to amplify the full sequence of sITLN2, with the sITLN3 specific reverse primer, sITLN R9-3. Specific primers for sITLN1, 2 and 3 had been designed for a poorly conserved region towards the 3' end (Table 2.4, Figure 2.5). The full sequence of sITLN3 was obtained from the overlapping sequences of the 5' and 3' ends and was verified in jejunal mucosa from two sheep using the especially designed full length primers sITLN F7 and R10. Using web based programmes the predicted open reading frame was identified, homology ascertained, and predicted signal peptides and N-glycosylation sites determined as described previously (2.3.13.6). The full sequence (1153 bp) thus obtained has a predicted opening reading frame of 972 bp and deduced amino acid sequence, 323 AAs (GenBank accession number AM888394). The full sequence shares 73% homology with hITLN1 (GenBank accession number NM017625), 86% homology with sITLN1 and 93% homology with sITLN2. The deduced amino acid sequence of sITLN3 shares 79% homology with hITLN1, 85% homology with sITLN1 and 91% homology with sITLN2. A 26 AA predicted signal peptide was identified (probability 0.992), with a predicted cleavage site (probability 0.509) between AA 26 and 27 (SAA-GT). No predicted N-glycosylation sites were identified using the programme NetNGlyc 1.0 despite the presence of a similar sequence to sITLN2 at AA 286 (Figure 4.1). There was no evidence of a GPI anchor site. Cysteine residues implicated in oligomer formation are shown in Figure 4.1B.

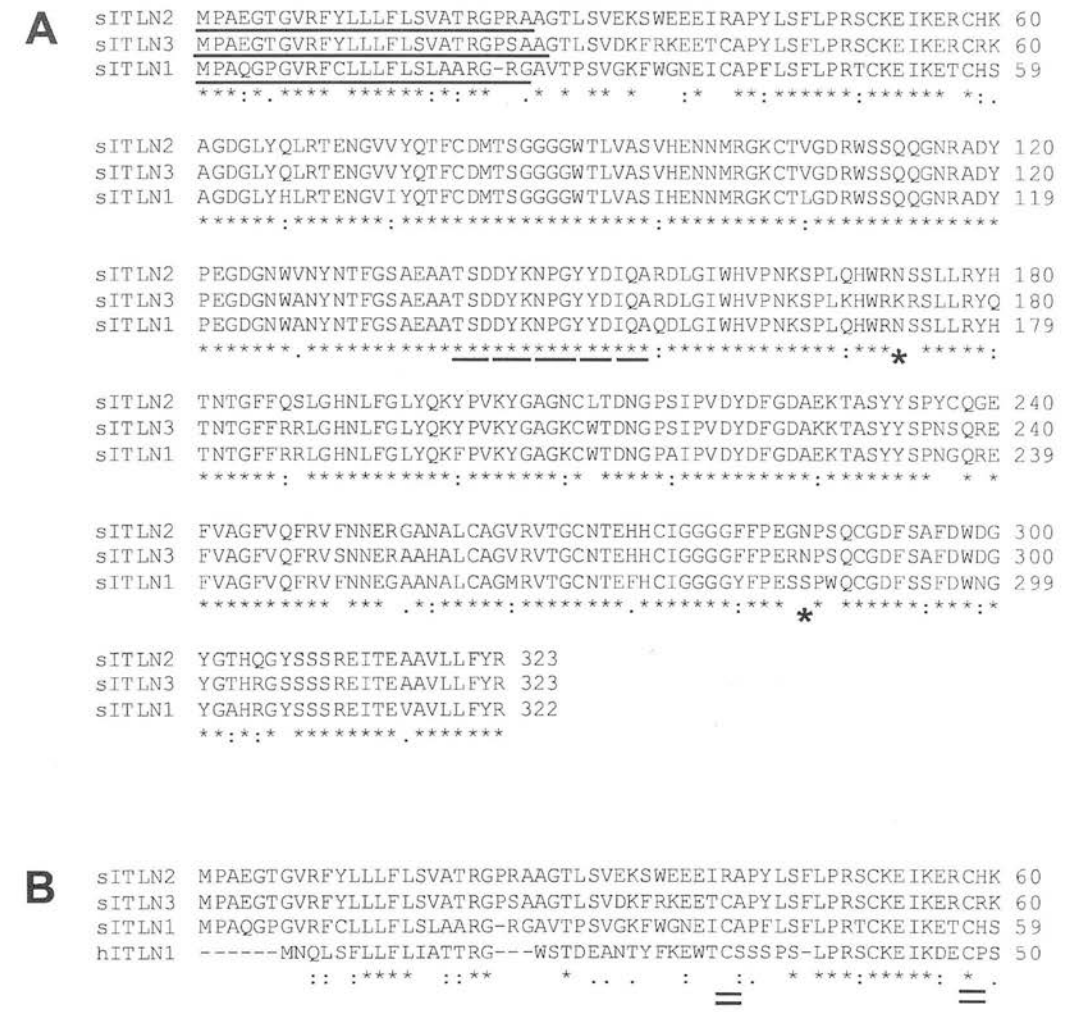


Figure 4.1: Alignment of amino acid sequences ITLN

(A) Alignment of predicted open reading frame of sheep ITLN (sITLN) 1, 2 and 3. Solid underlines delineate the predicted signal peptides, large asterix predicted N-glycosylation sites. The broken underline represents the peptide to which the affinity purified antibody was raised. Small asterices, single and double dots represent degree of homology.

(B) Alignment of predicted open reading frame of N-terminal region of sITLN1, 2 and 3 and N-terminal region of hITLN1. The double underline indicates the location of the cysteine residues that have been implicated in oligomer formation in hITLN1.

4.3.4 Cloning and sequencing of sheep SIAT4C

The full sequence of sSIAT4C was deduced from ESTs and confirmed using specific primers sSIAT4C F4R4, F5R5 and F6R6 in abomasal mucosa from 3 sheep as described in Material and Methods (2.3.16). Using web based programmes the predicted open reading frame was identified, homology ascertained, and predicted signal peptides, N-glycosylation sites and GPI anchor determined as described previously (2.3.13.6). The confirmed full sequence (1477 bp) thus obtained had a predicted opening reading frame of 1002 bp and deduced amino acid sequence, 333 AAs (Genbank accession number AM888395). The full sheep sequence shares 83% homology with human SIAT4C and 96% homology with bovine SIAT4C. The deduced amino acid sequence, shares 88 % homology with human SIAT4C and 99 % homology with bovine SIAT4C. A 26 AA predicted signal peptide was identified as seen in bovine and human SIAT4C and three predicted N-glycosylation sites were identified at AA 61, 131 and 310 in contrast to four in humans. There was no evidence of a GPI anchor. Cysteine residues were present at AA119 and 27 which have previously been shown to be highly conserved between species (Figure 4.2A). When deducing the full sequence of sSIAT4C, two different sheep ESTs (828727, 823877) were found for the 5' end. The one most similar to the bovine sequence was chosen and this sequence was confirmed in the abomasal mucosa. The alignment of sheep ESTs 828727, 823877 with sSIAT4C, bSIAT4C and hSIAT4C are shown in Figure 4.2B

Figure 4.2: Alignment of amino acid sequences SIAT4C

A. Alignment of predicted open reading frame of sheep SIAT4C (sSIAT4C) with bovine (b) SIAT4C and human (h) SIAT4C. Solid underlines delineate the predicted signal peptides, double underlines predicted N-glycosylation sites, hatch the predicted fourth N glycosylation site in hSIAT4C, plus sign conserved cysteine residues. The large asterices above the alignment represent the three amino acids which differ between the sheep and bovine sequences. Small asterices, single and double dots represent degree of homology.

B. Alignment of 60 base pair (BP) sequence of sSIAT4C with hSIAT4C, bSIAT4C, sheep expressed sequence tag (EST) 823877, 828727 at the 5' end showing 12 BP deletion present in sheep EST 828727 and hSIAT4C.

4.3.5 Absence of RELMβ in sheep

Using primers designed for human and mouse sequences, sheep respiratory and abomasal tissue was probed for evidence of RELMβ. There was no evidence of expression. Further work undertaken by Pam Knight using degenerate primers in different tissues failed to demonstrate a RELMβ homologue in sheep.

4.3.6 sITLN1 transcript expression in different tissues

Expression of sITLN1 transcript was examined in multiple tissues from normal sheep (MRI trial 3, group 1). Whilst samples were available from three sheep (numbered 33, 920, 1379) for most tissues, all tissues were not available from all three sheep. Expression was strongest in abomasums, colon, rectum and lung. Expression was shown in abomasum, lung, colon and gastric lymph node of 3/3 sheep, in terminal rectum of 2/3 sheep and in skin 1/3 sheep. Weak expression was also shown in jejunum 1/3, mesenteric lymph node 2/3, ileal peyer's patches 1/3, brain 1/3, kidney 2/3, liver 1/3, spleen 1/3, skin 1/3, ear pinna 1/3, heart 1/2 and ovary 1/1. There was no evidence of expression in uterus 1/1, pancreas or mesenteric fat 1/1. There was no expression of pancreatic ATPase in sheep 33 and 920 and it was presumed that this tissue had auto-digested prior to storage (Figure 4.3).

4.3.7 sITLN2 transcript expression in different tissues

Expression of sITLN2 transcript was examined in multiple tissues from three normal sheep (MRI trial 3, group 1). Transcript expression was only shown in the abomasum. Sheep 920 showed very weak expression of sITLN2 whilst sheep 33 and 1379 showed strong expression (Figure 4.3).

4.3.8 sITLN3 transcript expression in different tissues

Expression of sITLN3 transcript was examined in multiple tissues from normal sheep (MRI trial 3, group 1). Expression differed between sheep. Whilst samples were available from three sheep for most tissues, for some tissues samples were only available from one or two sheep. Sheep 920 showed a very different distribution pattern to the other two sheep with no expression of sITLN3 in any tissue. Strong expression was shown in abomasum, jejunum, colon, rectum and ileal peyer's patches in 2/3 sheep. Weak expression was also shown in gastric lymph node 2/3, mesenteric lymph node 2/3, kidney 2/3, liver 1/3, lung 2/3, spleen 2/3, skin 2/3, ear pinna 2/3, heart 1/2, ovary 1/1 and mesenteric fat 1/1. There was no evidence of expression in brain, pancreas 1/1 or uterus 1/1. As mentioned previously there was no expression of pancreatic ATPase in sheep 33 and 920 and it was presumed that this tissue had auto-digested prior to storage (Figure 4.3).

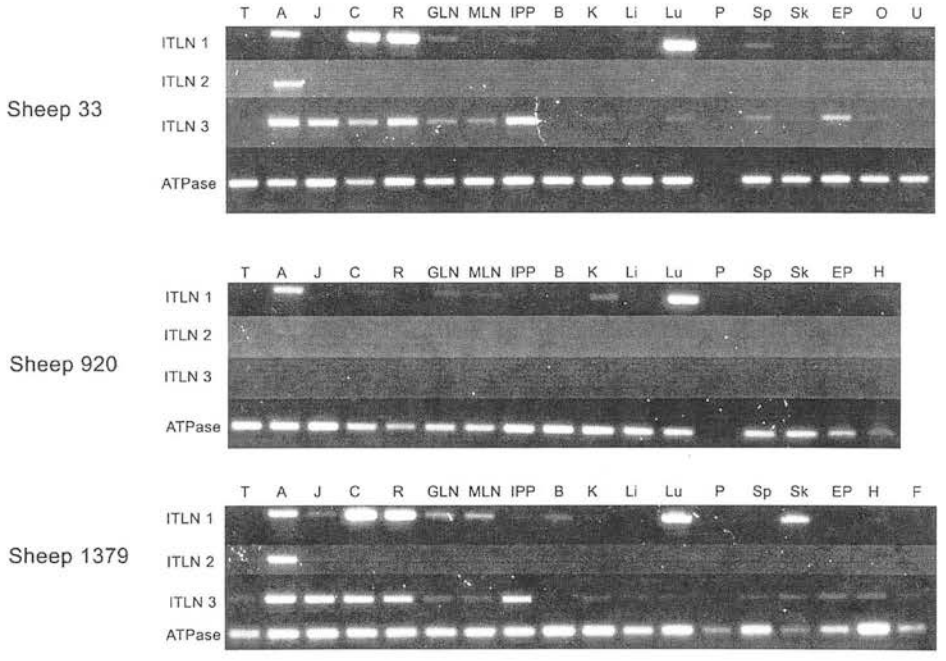


Figure 4.3: Tissue distribution of sheep ITLN transcripts

Tissue distribution of sheep ITLN (sITLN) 1, 2 and 3 transcript in three sheep (numbered 33, 920 and 1379). Expression of the house keeping gene ATPase is shown for comparison. Tongue (T), abomasum (A), jejunum (J), colon (C), rectum (R), gastric lymph node (GLN), mesenteric lymph node (MLN), intestinal peyer's patches (IPP), brain (B), kidney (K), liver (Li), lung (Lu), pancreas (P), spleen (Sp), skin (Sk), ear pinna (Ep), ovary (O), uterus (U), heart (H), fat (F).

4.3.9 Tissue distribution of sheep SIAT4C transcript

SIAT4C transcript was found to be expressed in the following tissues from the two sheep examined: abomasum, lung, colon, rectum, skin, jejunum, gastric lymph node, mesenteric lymph node, ileal peyer's patches, brain, kidney, liver, spleen, skin, ear pinna. In sheep 1379 transcript expression was also shown in pancreas, heart and fat (Figure 4.4).

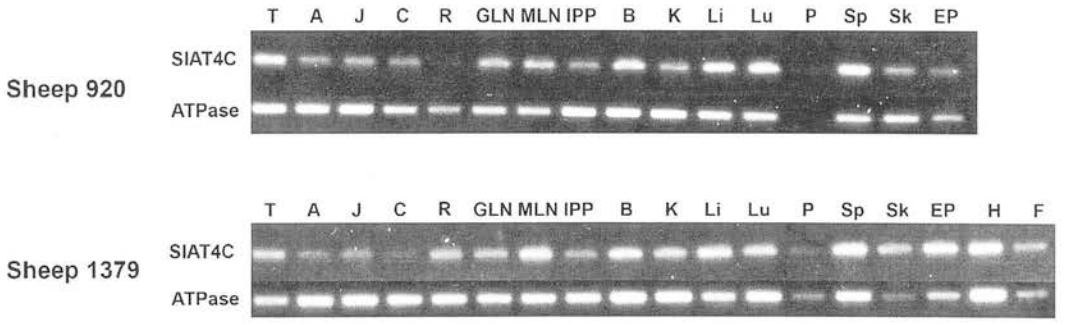


Figure 4.4: Tissue distribution of sheep SIAT4C

Tissue distribution of SIAT4C transcript in two sheep. Expression of the house keeping gene ATPase is shown for comparison. Tongue (T), abomasum (A), jejunum (J), colon (C), rectum (R), gastric lymph node (GLN), mesenteric lymph node (MLN), brain(B), kidney (K), liver (Li), lung (Lu), pancreas (P), spleen (Sp), skin (Sk), ear pinna (Ep), ovary (O), uterus (U), heart (H), fat (F).

4.3.10 Immunolocalisation of sheep ITLN

Multiple tissues from sheep 1379 (trial 3, group 1) were immunolabelled using the affinity purified chicken antibody to ITLN peptide 1 as described in Material and Methods (2.6.1 -2.6.2). Negative controls were immunolabelled with chicken IgY. There was no staining of negative controls. There was positive staining of the mucus neck cells in the abomasum (Figure 4.5A), the mucus cells in the colon (Figure 4.5B), free mucus in ileum (Figure 4.5D) and the goblet cells in the lung (Figure 4.5E). There was also staining of the small intestinal epithelium and brush border (Figure 4.5C). There was weak staining of the epidermal layer of the skin (Figure 4.5F), epithelium lining skin acinar glands (Figure 4.5G), skin sebaceous glands (Figure 4.5H), and of mesenteric fat (Figure 4.5I). There was no staining of heart muscle or heart fat (Figure 4.5J). There was no staining of lymph nodes or kidneys.

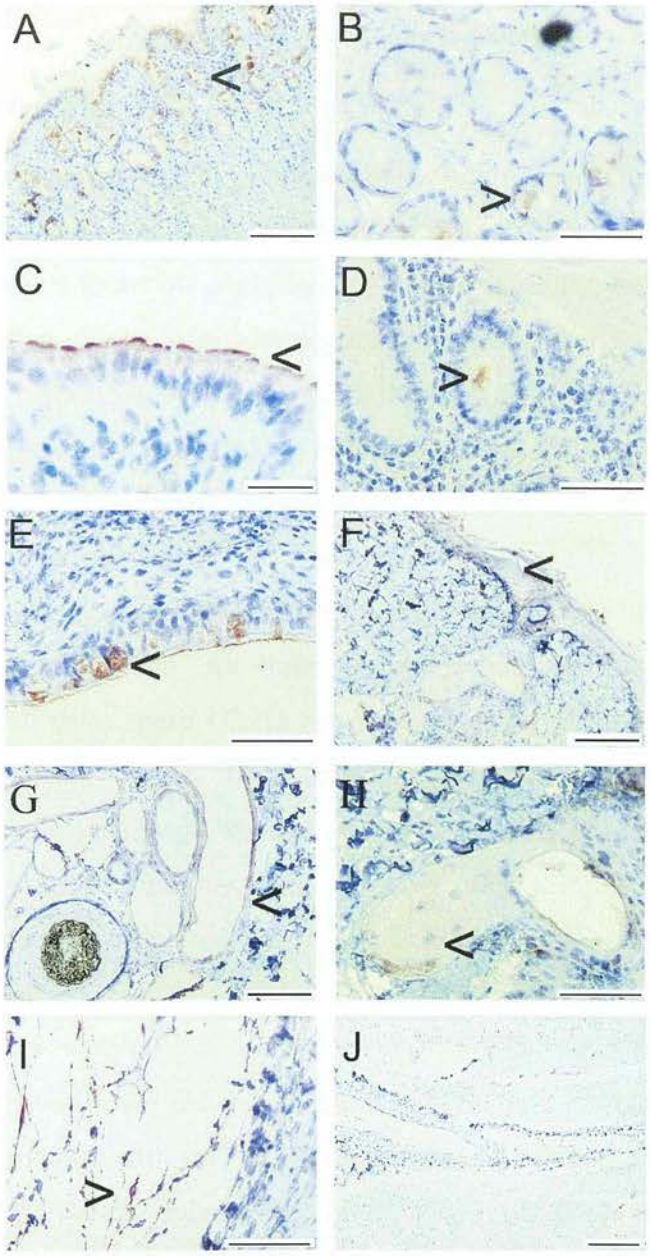


Figure 4.5: Immunolocalisation of sheep ITLN

Immunoperoxidase labelling of sections with affinity purified chicken anti-ITLN peptide 1. Abomasum (A), colon (B), small intestine (C), peyer's patches and ileum (D), large bronchus (E), dermis/epidermis (F), skin acinar gland (G), skin sebaceous gland (H), mesenteric fat and lymph node (I), heart (J). Arrows indicate positive immunolabelling Myocardium and fat within the heart wall was negative (J). Control IgY labelling was negative (not shown). Bars represent 25 μm (C), 50 μm (B, C, D, E, H), 100 μm (A, F, G), 200 μm (J).

4.4 Discussion

4.4.1 ITLN homologues

ITLNs appear to have arisen early in chordate evolution, and homologues are known in sea squirts, fishes (Berry et al., 2006; Gerwick et al., 2007), frogs (Chang et al., 2004) and mammals (Komiya et al., 1998; Khan et al., 2001; Kogure et al., 2006). Within mammalian species two homologues have been described in mice, humans and recently in cattle (Stacey Blease, MSc thesis, University of Edinburgh 2007). The present study has demonstrated the presence of three ITLN homologues in sheep which shared 85 – 91% homology with each other and 53 – 81% homology with human and murine sequences. For the three sheep ITLNs the predicted AA sequences were poorly conserved at the N-terminus and better conserved towards the middle and C-terminus end. All three sequences had predicted signal peptides, suggestive that all three sheep ITLNs can be secreted, however only sITLN2 had a predicted GPI anchor site at the C-terminus. ITLN has been recognised as a lactoferrin receptor in several species and the different forms of ITLN in the intestinal brush border may explain the very diverse functions attributed to lactoferrin including nuclear transcription and cell surface bacteriostasis (Wrackmeyer et al., 2006).

Recent studies have shown that recombinant hITLN1 is a 120kDa trimer, however despite 84% homology with mITLN1, recombinant and intestinal mITLN1 exist as unglycosylated 30kDa monomers (Tsuji et al., 2007). In the present study sITLN1 and sITLN3 have Cys-39/40 and Cys-57/58 respectively which align with human Cys-31 and Cys-48 that have been implicated in oligomer formation, however sITLN2 differs and has only got a Cys-58. Again this may suggest a difference in structure and possibly function for sITLN2 compared to sITLN1 and sITLN3. Whilst no work has been undertaken to examine the carbohydrate specificity of the sheep ITLNs, it cannot be presumed that they are identical to other species as recent work has shown that whilst hITLN1 and mITLN1 preferentially bind a galactonfuranosyl- compared to a galactopyranosyl-containing saccharide, they have different saccharide binding specificities from each other (Tsuji et al., 2007).

The three sheep ITLNs differed in the number of predicted N-glycosylation sites with one present in sITLN1, 2 in sITLN2 and none in sITLN3. This is another factor that might suggest different functional roles for the three sheep ITLNs.

4.4.2 ITLN expression

The three sheep ITLNs differed in transcript tissue distribution pattern, as has been reported previously for the two murine (Komiya et al., 1998; Pemberton et al., 2004a; Suzuki and Lonnerdal, 2004) and two human homologues (Lee et al., 2001). Transcripts for sITLN1 and sITLN3 were present in several tissues whilst sITLN2 transcript was restricted to the abomasum in the normal animal. For sITLN1, transcript expression was shown in abomasum, jejunum, colon, rectum, lung, heart, lymph node, ileal Peyer's patches, spleen, kidney, liver, skin, brain, ovary. Whilst for sITLN3 expression was shown in the same range of tissues with the exception of brain. There was no expression sITLN1 or sITLN3 in uterus, pancreas or mesenteric fat. In mice ITLN1a transcript has been shown in the intestine, lung, heart, spleen, kidney, and testis (Suzuki et al., 2005) whilst studies in man have shown expression of hITNL1 transcript in the stomach, small intestine, colon, lung, heart, lymph node, spleen, thymus, ovary, testis (Lee et al., 2001). As found for sITLN2, transcripts for hITLN2 (Lee et al., 2001) are restricted to the gastrointestinal tract whilst for mITLN1b/2 expression is restricted to the respiratory tract in the normal animal (Pemberton et al., 2004a; Voehringer et al., 2007).

The variation in expression of the three ITLNs between sheep was of particular interest, with sheep 920 demonstrating no expression of sITLN2 and 3 transcripts in any tissue. It is known that some in-bred murine strains show genomic variation, with the *T. spiralis* strain that expels worms slowly, C57BL10, lacking mITLN1b/2 (Pemberton et al., 2004a). It is unknown if similar variation occurs in sheep or people and the significance of this finding regarding expression patterns of disease. Further genomic studies are required to determine if there is genomic variation in expression of ITLNs between sheep as seen with mice.

In the present study sITLN was immunolocalised to epithelial cells and mucus producing cells with expression shown in the gastro-intestinal tract, respiratory tract and skin of normal sheep. Whilst strong immunolabelling was present in mucus producing cells of the abomasum and colon, and goblet cells in the respiratory tract, protein expression was also present on the small intestinal brush border as has been reported for mice and pigs (Suzuki and Lonnerdal, 2004; Wrackmeyer et al., 2006). There was also faint immunolabelling of the enterocytes as reported in pigs (Wrackmeyer et al., 2006). These findings are suggestive that ITLN is not only produced by mucus producing cells. In mice, ITLN has been immunolocalised to a greater range of tissues with expression reported in gastro-intestinal, respiratory, reproductive, urinary tract, lymphatic, nervous and endocrine tissue (pancreas) (Suzuki and Lonnerdal, 2004) whilst in man protein expression has been shown in endothelial cells, respiratory epithelial cells, mesothelial cells, omental fat and serum (Yang et al., 2006).

The presence of ITLN transcript and protein in sheep skin tissue is very interesting as there are no reports of expression of ITLN in skin of other mammalian species. ITLN appeared to be immunolocalised to sebaceous glands, the epithelial cells lining the apocrine glands and to the dermis. Expression of lactoferrin has been shown in the skin in man (Cumberbatch et al., 2000; Ward et al., 2002) and furthermore has been shown to bind to keratinocyte epithelial cells using competitive binding assays. This finding supports the presence of lactoferrin receptors in keratinocytes (Ward et al., 2002). ITLN may play an independent role in skin defence or have a role as a lactoferrin receptor in this tissue.

ITLN1 and 3 transcripts were shown in lymphoid tissue in the sheep however there was no convincing evidence of protein expression on immunohistochemistry. Studies in mice have shown immunolabelling of small lymphocytes in the lymph node and spleen (Suzuki and Lonnerdal, 2004). In man, whilst hITLN1 transcript was shown in lymph node, spleen and thymus, protein expression was immunolocalised to endothelial cells (Lee et al., 2001). Interestingly in carp, whilst there was

immunolabelling of endothelial cells in the spleen, small lymphocytes were also immuno-labelled as well as clusters of immunoreactive cells (macrophages/lymphocytes) in the kidney (Chang and Nie, 2007). Thus, there is evidence for a role for ITLN in lymphoid tissue and further work may clarify this role.

Omentin, shown to be identical to hITLN1, is thought to have a link to obesity and type II diabetes mellitus and expression has been shown in mesenteric fat cells (Schaffler et al., 2005; Yang et al., 2006; Wurm et al., 2007). In the present experiment there was evidence of sITLN3 transcript expression in mesenteric fat tissue and slight immunolabelling of mesenteric fat. Further work is required to verify these findings. Immunolabelling of neurological tissue has been shown in mice and in carp ((Suzuki and Lonnerdal, 2004; Chang and Nie, 2007) however whilst sITLN1 transcript was shown in brain from sheep 1379 in the present experiment, there was no evidence of immunolabelling of cells. This disparity may reflect a species difference or difference in immunolabelling techniques or part of the brain examined.

4.4.3 SIAT4C sequence

The SIAT4C sequence appears highly conserved between species and in the present study sSIAT4C was shown to share 88 - 99% homology with other mammalian sequences. The presence of a predicted signal peptide would support secretion of this molecule. The conserved N glycosylation sites and cysteine residues found between species might suggest that structure and function may also be conserved between species. Previous studies have shown the presence of several isoforms of SIAT4C in man (Kitagawa and Paulson, 1994b; Grahn and Larson, 2001). In the present study, no attempt was made to determine if more than one isoform was present in sheep. However, interestingly a sheep EST with a 12 bp deletion at the same site as the deletion found in human isoforms B-12, A1-12, A2-12 was found on GenBank, which suggests that isoforms are present in sheep. Further work is required to confirm this finding.

4.4.4 SIAT4C expression

Previous work has shown wide tissue expression of SIAT4C in mice and man (Grahn and Larson, 2001; Ellies et al., 2002a). In the present study SIAT4C transcript expression was shown in abomasum, lung, colon, rectum, skin, jejunum, gastric lymph node, mesenteric lymph node, ileal peyer's patches, brain, kidney, liver, spleen, skin, ear pinna, pancreas, myocardium and colon. These findings are in agreement with previous reports for mice and man (Grahn and Larson, 2001; Ellies et al., 2002a). Unfortunately no reproductive tract tissues were available for the two sheep examined however transcript expression has been shown in the human ovary, testes, placenta, uterus and prostate (Grahn and Larson, 2001) and in mouse ovary, testes and placenta (Ellies et al., 2002a) and it is likely that in sheep, these tissues may also be found to express SIAT4C. The nine alternatively spliced SIAT4C transcripts described in man were shown in peripheral blood leukocytes (PBL). It is unknown if SIAT4C is expressed in sheep PBLs (Grahn and Larson, 2001).

Whilst transcript expression has been shown in many tissues, there are no reports in the literature on the immuno-localisation of SIAT4C expression in different species. There are few antibodies available and those available are not suitable for use in sheep. Alternatively, immuno-localisation could be considered using the lectin, Maackia Amurenis (MAL 11), which recognises sialic acid linked α2-3 to Galβ1-4GlcNAc, Galβ1-3GlcNAc and Galβ1-4GalNAc. However, as at least five sialyltransferases are responsible for α2-3 linkages in man, and the same may be true for sheep, thus MAL II cannot be considered as a specific marker of SIAT4C (Varki et al., 1999). Therefore SIAT4C protein expression was not demonstrated in sheep.

4.4.5 RELMβ expression

RELMβ primers suitable for sheep and human tissue did not result in amplification of a product from sheep tissues. Further work was undertaken by Pam Knight using degenerate primers to determine if any of the RELM family member transcripts were

expressed in sheep, but only Resistin was found expressed in sheep tissues examined (unpublished data).

4.5 Conclusion

Three ITLNs and at least one SIAT4C transcript are expressed in sheep. sITLN transcripts differ in tissue distribution. sITLN1, sITLN3 and sSIAT4C show wide tissue distribution whilst sITLN2 is restricted to the abomasum in normal sheep. ITLN protein is present in the gastro-intestinal and respiratory tract and skin, and is immunolocalised to mucus producing cells and epithelium. The presence of ITLNs and SIAT4C in the gastro-intestinal and respiratory tract of sheep will allow further study of these molecules in a Th2 environment in sheep.

5. Expression of ITLN and SIAT4C in the respiratory tract of sheep

5.1 Summary

sITLN1, sITLN3 and SIAT4C were found to be constitutively expressed in the trachea and lungs of normal sheep. Incubation of sheep tracheal explants or sheep respiratory cell cultures with Th2 cytokines or infection with *Dictyocaulus filaria* resulted in expression of all three sITLNs in respiratory tissue. Infection with *D. filaria* caused induction of sITLN2 and significant upregulation of sITLN3. Incubation of sheep tracheal explants with recombinant sIL-4 resulted in significant downregulation of SIAT4C which did not appear to be associated with increased neutral mucins. sITLNs and SIAT4C appear to be regulated differently by Th2 cytokines.

5.2 Introduction

In man, expression of transcript for the mucus associated molecule ITLN has been show in respiratory tract epithelium, with upregulation occurring in response to culture with the Th2 cytokine IL-13 (Kuperman et al., 2005). Transcript expression has also been shown in bronchoalveolar lavage fluid of asthmatic individuals (Kuperman et al., 2005). In mice upregulation of mITLN1a and mITL1b/2 transcript has been shown in the lungs of mice in response to *N. brasiliensis* infection known to induce a typical Th2 response (Voehringer et al., 2007). Expression of SIAT4C transcript, another mucus associated molecule, has been shown in lung tissue of mice (Ellies et al., 2002a) and humans (Grahn and Larson, 2001). Sheep are important models for respiratory tract disease in man (Abraham et al., 1999; Bischof et al., 2003; Abraham et al., 2005; Snibson et al., 2005) and in chapter 4 expression of

sITLN1, sITLN3 and sSIAT4C transcripts was shown in lung tissue from normal sheep. Whilst mITLN1b/2 and mSIAT4C are both known to upregulate in the gastrointestinal tract in response to nematode infections which produce a typical Th2 response ((Knight et al., 2004; Pemberton et al., 2004a; Datta et al., 2005; Artis, 2006; Yamauchi et al., 2006; Kawai et al., 2007; Voehringer et al., 2007), results of cell cultures in chapter 3 would suggest that these molecules are regulated by different mechanisms. The following experiments were undertaken to further examine the co-regulation of these molecules in the respiratory tract using sheep as a model. Source of materials, method for tissue collection and preparation, molecular biology techniques, imunnohistochemistry and histochemistry protocols have been described fully in chapter 2. A Mann-Whitney U test was used for all paired and unpaired comparisons.

5.3 Results

5.3.1 Expression of ITLN and SIAT4C in lungs and trachea from normal sheep

5.3.1.1 *Confirmation of normality*

Paraformaldehyde fixed paraffin embedded haematoxylin and eosin stained sections of trachea and lungs from six sheep (MRI trial 2, group 1) raised in an environment to exclude accidental infection with parasites, were examined under light microscopy and found to be normal. Bronchoalveolar lavage samples taken at post mortem from these six sheep all showed a high percentage of macrophages as has been described in BAL fluid from normal sheep (Figure 5.1). There were less than 5% lymphocytes and neutrophils in the majority of sheep except in two samples where blood contamination led to slightly higher neutrophil counts. Eosinophil counts were always < 2%. Full details of materials and method are described in chapter 2 (2.2.9).

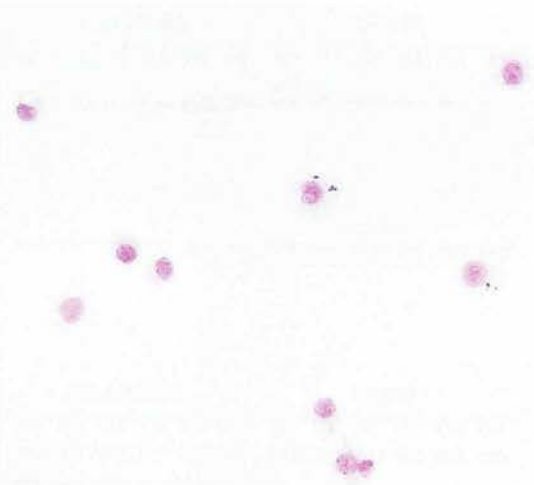


Figure 5.1: Cytospin of bronchoalveolar lavage fluid

Broncho-alveolar lavage from one sheep showing predominantly macrophages.

5.3.1.2 *ITLN and SIAT4C transcript expression in trachea and lung from normal sheep*

Using RT-PCR, transcripts for the three sITLNs were shown to differ in their expression pattern in lung and trachea from the six normal sheep. Full details of method and primers used are given in chapter 2 (2.3.1.2, 2.3.4 – 2.3.7, 2.4.3).Whilst sITLN1 and sITLN3 showed constitutive expression in both trachea and lung, sITLN2 showed limited expression and was only shown in trachea from one sheep (U1). Of interest was the apparent stronger expression of sITLN1 and sITLN3 in trachea compared to lung. There was strong constitutive expression of SIAT4C transcript in sheep lung and trachea and interestingly the expression pattern differed to sITLNs with apparent stronger expression in lungs compared to trachea (Figure 5.2).

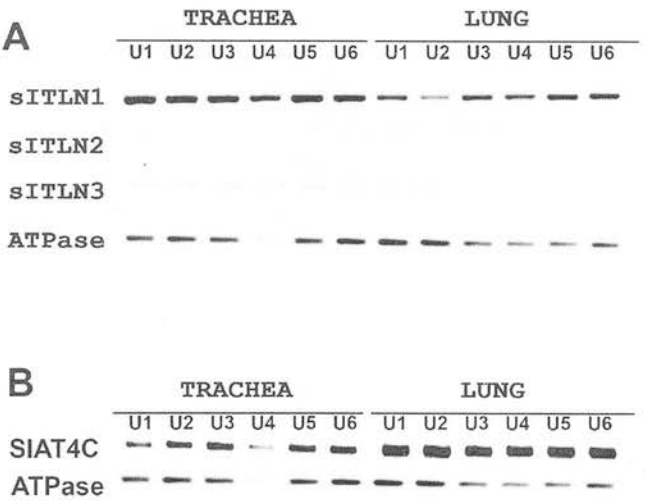


Figure 5.2: Ethidium bromide gel showing transcript expression

Expression of sITLN1, sITLN2 and sITLN3 transcripts (A) and SIAT4C transcripts (B) in trachea and lungs from six normal sheep labelled U1-U6. Expression is shown compared to the housekeeping gene sheep ATPase.

5.3.1.3 *ITLN protein expression in trachea and lung from normal sheep*

Following optimisation of the immunohistochemistry technique (2.6.1 – 2.6.2) trachea and lung sections were immunolabelled with two different ITLN antibodies, affinity purified chicken anti-ITLN peptide 1 and affinity-purified polyclonal rabbit against *Xenopus* oocyte lectin XL35 (anti-XL35). The affinity purified anti-ITLN peptide 1 resulted in strong constitutive expression of ITLN protein in all trachea and lung sections examined from the six normal sheep. Expression was present in goblet cells, submucosal glands and surface mucus. On the other hand the anti-XL35 antibody immunolabelled sections from sheep U1-U3 strongly but showed limited staining in sheep U4 - U6. Immunohistochemistry was repeated to make sure the results were repeatable. Interestingly the anti-XL35 antibody consistently stained surface mucus/glycocalyx better than anti-ITLN peptide 1 (Table 5.2 and Figure 5.3).

	Trachea	Large bronchus	Small bronchus
	GC/SMG/FM	GC/SMG/FM	GC/FM
Sheep U1			
Anti-Peptide 1	+/+/-	++/-/-	++/-
Anti-XL35	+/++/+++	++/++/+++	-/++
Sheep U2			
Anti-Peptide 1	++/+/-	+++/+/+++	+/+
Anti-XL35	++/++/+++	++/++/+++	-/++
Sheep U3			
Anti-Peptide 1	++/+/-	++/-/-	++/-
Anti-XL35	+/-/+	+/-/+	-/-
Sheep U4			
Anti-Peptide 1	++/+/-	+++/+/++	++/++
Anti-XL35	-/-/-	-/-/-	-/-
Sheep U5			
Anti-Peptide 1	++/+/++	++/+/++	++/++
Anti-XL35	-/-/-	-/-/+	-/-
Sheep U6			
Anti-Peptide 1	+++/+/-	+++/+/++	+/-
Anti-XL35	-/-/-	+/-/-	-/-

Table 5.1: Immunolabelling of normal sheep respiratory tissue

Comparison of immunolabelling, with anti-ITLN peptide I and anti-XL35 antibodies, in trachea and lung section from six normal sheep. Goblet cells/ submucosal glands/ free mucus (GC/SMG/FM). The negative sign denotes no immunolabelling and plus signs represent strength of immunolabelling (+ weak, ++ stronger, +++ strongest).

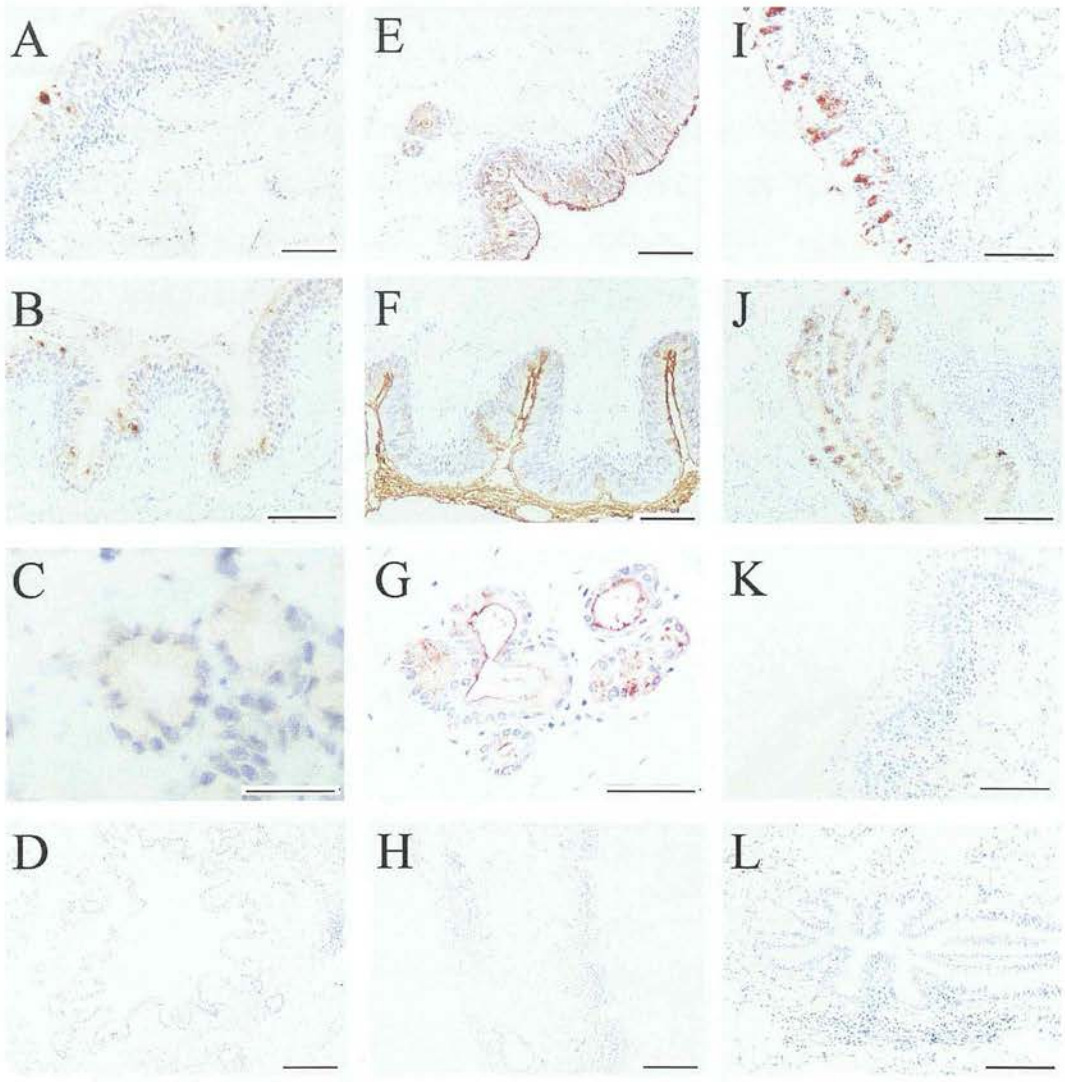


Figure 5.3: Immunolabelling of respiratory tract in normal sheep

Immunolabelling with affinity purified anti-ITLN peptide 1 antibody in respiratory tract from sheep U2 (A, B, C, D) showing trachea, large airway, submucosal gland and control IgY respectively. Immunolabelling with anti-XL35 antibody in sheep U2 (E, F, G, H) showing trachea, large airway, submucosal gland and control IgG respectively. The positive immunolabelling with anti-peptide 1 antibody (I, J) and negative immunolabelling with anti-XL35 antibody (K, L) in sections from sheep U4 are illustrated. Note the staining of the glycocalyx/brush border with XL35 anti-ITLN antibody in E, F and G. Bars are 50 μm (C, G), 100 μm (B, I, J, K, L), 200 μm (A, D, E, F, H).

5.3.1.4 Alcian blue/periodic acid Schiff histochemistry in trachea and lung from six normal sheep

No suitable antibody was available for immunolabelling of SIAT4C. SIAT4C results in acidification of mucins by transfer of sialic acid and thus as a method of demonstrating the presence of acidic mucins, sections were stained with alcian blue /periodic acid Schiff which labels acidic and neutral mucins, torquoise and magenta respectively. There was staining of neutral (magenta) and acidic (turquoise) mucins in the goblet cells, submucosal glands and on the glycocalyx. Neutral mucins predominated in the submucosal glands. The glycocalyx/brush border stained acidic. There was no difference noted between sheep (Figure 5.4).

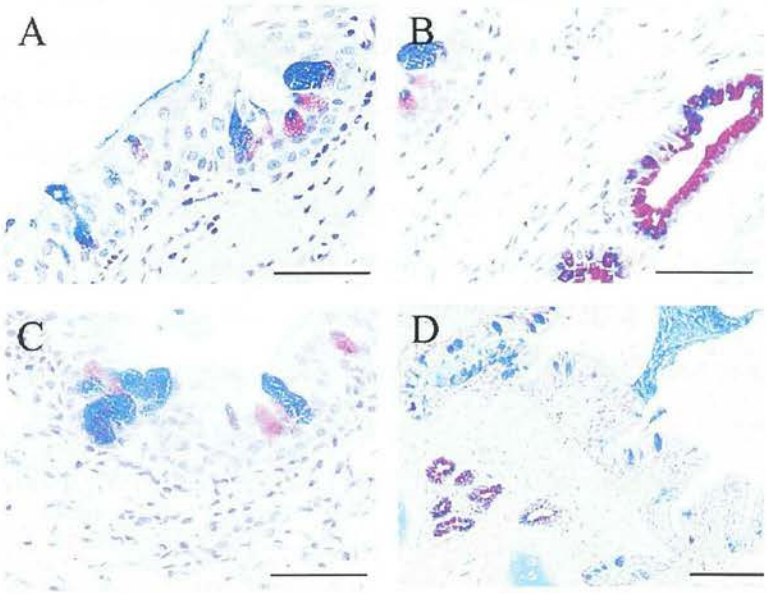


Figure 5.4: Alcian blue/periodic acid Schiff histochemistry of respiratory tract in normal sheep

Alcian blue/ periodic acid Schiff histochemistry of trachea (A, B) and large bronchus (C, D). Note the acidic glycocalyx/brush border and the presence of neutral, acidic and mixed mucins in the tracheal goblet cells, bronchial goblet cells and sub-mucosal glands. Bars are 50 μm (A, B, C), 100 μm (D).

5.3.2 Sheep tracheal explants as a model to examine response to the Th2 cytokine IL-4

5.3.2.1 Introduction

Work undertaken with Julie Bethune (MSc Thesis, University of Edinburgh 2005) in 2005 had shown up-regulation by real time RT-PCR of ITLN transcript in sheep tracheal explants maintained in medium containing recombinant sheep IL-4 (sIL-4) for 48 hours compared to medium only controls (French et al., 2007). As consensus ITLN primers had been used it was unknown if this upregulation was due to a specific sheep ITLN and furthermore it was unknown if there was upregulation of ITLN protein. Semi-quantitative RT-PCR was undertaken using specific primers for sITLN1, 2 and 3 and also ITLN protein expression was assessed using affinity purified anti-ITLN peptide 1 antibody in these explants. The expression of SIAT4C transcript was also determined using RT-PCR and alteration in character of pH of mucins assessed by enumeration of neutral and acidic staining goblet cells. Full details of tracheal explant protocol are given in chapter 2 (2.1.2, 2.2.3). There was concern that any increase in gene or protein expression might simply be a result of better preservation of epithelium in the presence of IL-4. Thus H&E sections were assessed by an independent board certified pathologist blinded to the identity of the sections, who found no difference in viability of the epithelium between samples. A paired Mann-Whitney U test was used for statistical analysis, a p-value < 0.05 was considered significant.

5.3.2.2 Expression of sITLN1, sITLN2, sITLN3 and SIAT4C transcripts in tracheal explants

Using RT-PCR and primers as described previously (2.3.1.2, 2.3.4 – 2.3.7, 2.4.3) the expression of transcripts for the three sITLNs and for SIAT4C was examined in tracheal explants incubated in medium only or medium plus sIL-4 for 48 hours from 5 sheep numbered 1379,118, 156, 251 and 419 (Figure 5.5). Transcript was expressed relative to the house keeping gene sheep ATPase. The expression of sITLN transcripts in tracheal explants maintained in medium only differed in these five sheep; 4/5 expressed sITLN1, 3/5 expressed sITLN2 and 3/5 expressed sITLN3.

The response to incubation with recombinant sIL-4 for 48 hours also varied considerably between sheep. For sITLN1 upregulation/induction was seen in 3/5 sheep, down regulation in 1/5 and no change in 1/5. For sITLN2 one sheep (419) showed no expression of transcript either in medium alone or following incubation with sIL-4 whilst the remaining 4 sheep all showed upregulation/induction however this failed to reach significance because of low numbers using a Mann-Whitney U test (p = 0.1297). For sITLN3 one sheep showed no expression (419), 3/5 sheep showed upregulation/induction and one sheep showed downregulation following incubation with sIL-4 compared to medium only controls. In comparison tracheal explants from all sheep showed downregulation of SIAT4C transcript following incubation with sIL-4 compared to medium which reached significance (p = 0.0041).

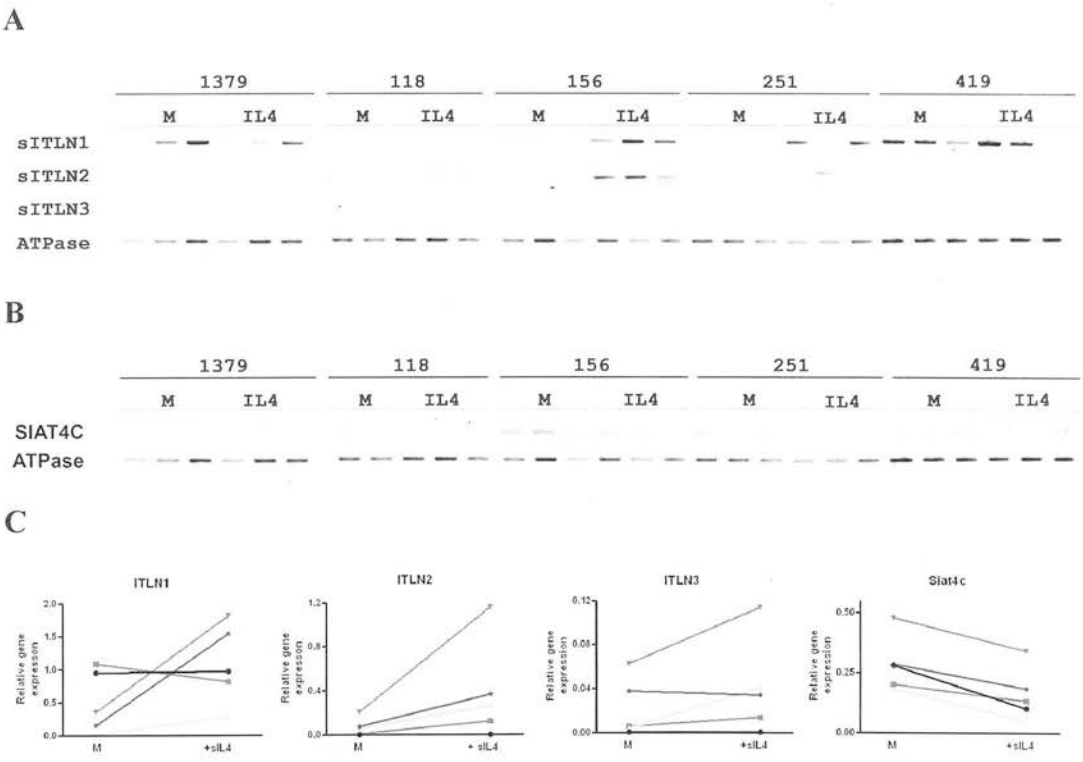


Figure 5.5: Transcript expression in sheep tracheal explants

Ethidium bromide gels showing relative expression of sheep ITLN1, sITLN2, sITLN3 (A) and SIAT4C (B) transcripts in sheep tracheal explants (three replicates) incubated in medium alone or in medium containing recombinant sheep IL-4 (sIL-4) and graphs showing relative gene expression of these transcripts (C). Sheep were labelled as follows: 1379,118, 156, 251, 419. Transcript expression is shown relative to the house keeping gene sheep ATPase. M = medium. The coloured lines represent

the following individual sheep: red (1379), yellow (118), green (156), blue (251), black (419). For sheep 118 only 2 replicates were available for medium only controls.

5.3.2.3 Expression of ITLN protein in sheep tracheal explants

Sheep tracheal explants were immunolabelled with anti-ITLN peptide 1 (2.6.1 – 2.6.2). There was positive staining of numerous goblet cells in the section take from explants before they were placed in medium (Figure 5.6B). Once placed in medium the goblet cells degranulated resulting in very few positive staining cells. There was a trend to an increase in ITLN positive goblet cell numbers in explants maintained in medium plus sIL-4 compared to explants in medium only however this failed to reach statistical significance using a Mann-Whitney U test (p = 0.1595). The cells were enumerated as described previously (2.7) and the results are shown in Figure 5.6A.

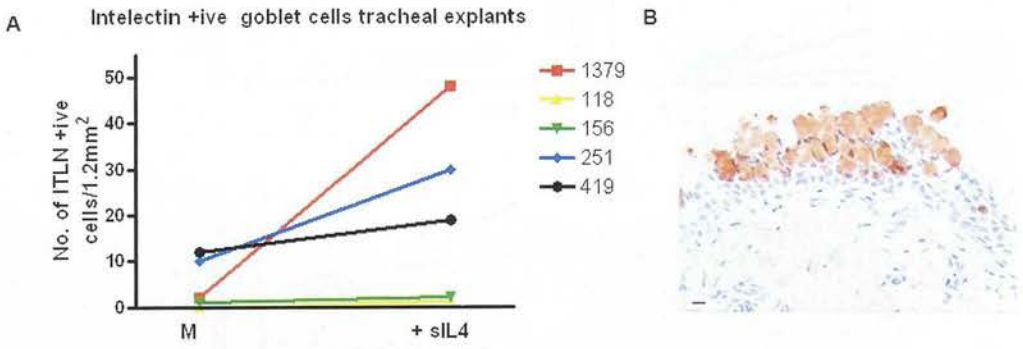


Figure 5.6: Immunolabelling of sheep tracheal explants

(A) Enumeration of goblet cells in sheep tract explants immunolabelling with anti-ITLN peptide 1 antibody. Number of goblet cells positive for intelectin (ITLN) following incubation for 48 hours in medium alone or medium + recombinant sheep IL-4 (sIL-4) (n=5) are shown. (B) ITLN immunolabelling of tracheal explant from sheep 251 before incubation.

5.3.2.4 Carbohydrate histochemistry - Alcian blue/ Periodic acid Schiff staining

Alcian blue/periodic acid Schiff histochemistry of sheep tracheal explants sections was carried out as described in chapter 2 (2.6.5). Goblet cells were shown to contain both acidic and neutral mucins which were numerous in explants before incubation in medium (Figure 5.7D). The goblet cells degranulated when incubated for 48 hours resulting in detection of fewer goblet cells compared to baseline, however the presence of recombinant sIL-4 in the medium resulted in either no change or an increase in the number of goblet cells detected using the combined AB/PAS stain at 48 hours ($p = 0.0607$). When PAS and AB positive cells were enumerated separately the number of AB positive cells either remained the same or increased in all sheep following incubation in medium containing recombinant sIL-4 compared to control ($p = 0.0898$) whilst number of PAS positive cells remained the same, increased or decreased ($p = 0.7483$).

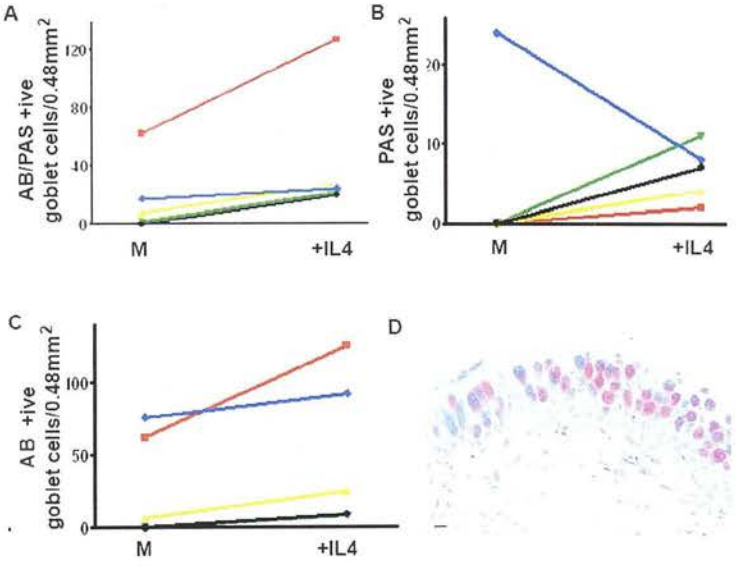


Figure 5.7: AB/PAS carbohydrate histochemistry of sheep tracheal explants

Number of Alcian blue (AB)/Periodic acid Schiff (PAS) positive goblet cells (A), PAS positive goblet cells (B) and AB positive goblet cells (C) in tracheal explants after culture in medium alone or medium + recombinant sheep IL-4 for 48 hours. AB/PAS staining of tracheal explant from sheep 251 before culture, showing neutral (magenta) and acidic (turquoise) mucins (D).

5.3.3 Sheep respiratory cell cultures as a model to determine expression of transcripts in response to incubation with Th2 cytokines IL-4 and IL-13

5.3.3.1 Introduction

In late 2007 a sheep respiratory cell culture model was established at the University of Edinburgh by Badrul Yahaya (PhD student). Previous work with this model had shown that good cell differentiation occurred within 3 weeks of culture (Personal communication, G. McLachlan). RNA was available from a limited number of cell cultures to examine expression of sITLN1, 2 and 3 transcripts in response to incubation with selected Th2 cytokines.

5.3.3.2 Incubation of respiratory cell cultures with Th2 cytokines

Using RT-PCR the expression of sITLN1, 2 and 3 transcripts and the house keeping gene, sheep ATPase, was examined in two cell cultures grown for 3 weeks in medium or medium containing either recombinant sIL-4 or recombinant human IL-13 (hIL-13). Full details of molecular biology techniques and primers have been described previously (2.3.1.2, 2.3.4 – 2.3.7, 2.4.3). There was strong constitutive expression of sITLN1 in 1/2 cultures and upregulation/induction occurred following incubation with either sIL-4 or hIL-13. There was weak constitutive expression of sITLN2 and sITLN3 in 1/2 cultures and incubation with either sIL-4 or hIL-13 resulted in upregulation/ induction of both sITLN2 and sITLN3.

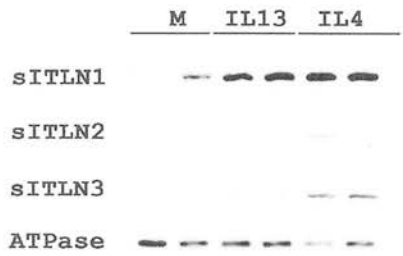


Figure 5.8: Transcript expression in sheep respiratory cell cultures

Ethidium bromide gel (1.4%) showing expression of sITLN1, sITLN2 and sITLN3 transcripts in sheep respiratory cell culture (2 replicates) following incubation with medium only or medium containing recombinant sheep IL-4 or recombinant human IL-13. Expression of the house keeping gene sheep ATPase is shown for comparison.

5.3.4 Expression of sheep ITLN transcripts and protein in sheep lung following natural infection with *Dictyocaulus filaria*

5.3.4.1 Introduction

Sheep are commonly affected with the lungworm *Dictyocaulus filaria* which may cause serious loss in productivity (Al-Sammarrae and Sewell, 1977; Bekele et al., 1992; Tembely et al., 1997; Alemu et al., 2006; Panuska, 2006). The cytokine response in sheep lungworm infection has not been defined, however the disease is characterised by eosinophil infiltration (Pfeffer, 1981) and is likely to be a predominantly Th2 response. In the following experiment ITLN transcripts were examined in lung tissue from yearling sheep naturally infected with the lung worm *Dictyocaulus filaria* and compared to the six normal sheep described in experiment 1 as controls. The objective was to determine if sITLN transcripts changed *in vivo* in a presumed Th2 environment as had been shown in response to culture with Th2 cytokines in the *in vitro* and ex vivo models (5.3.2, 5.3.3).

5.3.4.2 Results

The expression of transcripts for the three sITLNs was determined by RT-PCR in six *D. filaria* infected sheep and 6 control sheep using techniques described previously

(2.1.5, 2.3.1.2, 2.3.4 – 2.3.7, 2.4.3). Expression was determined relative to the house keeping gene sheep ATPase. sITLN1, sITLN2 and sITLN3 transcripts were expressed in lung samples from *D. filaria* infected sheep whilst only expression of sITLN1 and sITLN3 was shown in lungs from control sheep. There was no significant difference in expression of sITLN1 between infected and control sheep (p = 0.0649) whilst there was a significant difference in expression of sITLN2 (p = 0.0022) and sITLN3 (p = 0.0152) between infected and control sheep with induction of sITLN2 and upregulation of sITLN3 in the infected sheep (Figure 5.9).

Lung tissue sections from *D. filaria* infected sheep were immunolabelled with anti-ITLN peptide 1 antibody and were compared to lung tissue sections from six control sheep. No difference in expression of ITLN was seen between sections from infected and control sheep (not shown)

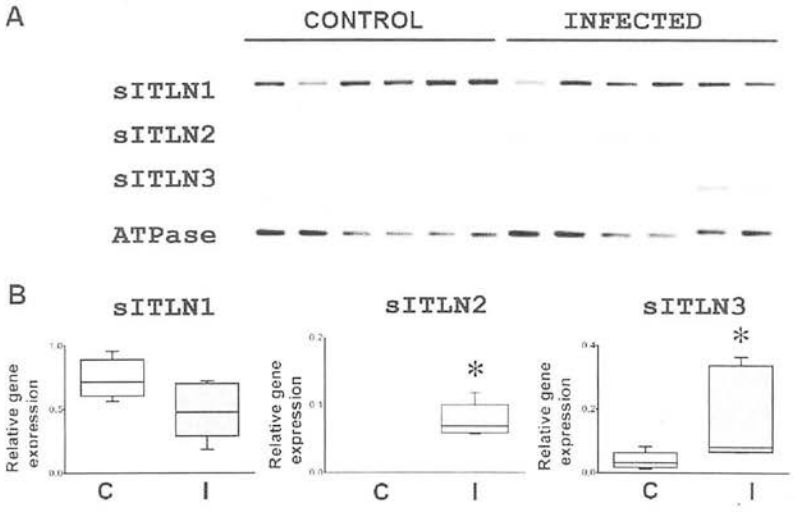


Figure 5.9: ITLN transcript expression in *Dictyocaulus filaria* infected sheep
Ethidium bromide gel showing relative expression of sITLN1, 2 and 3 transcripts in lung from normal and *Dictyocaulus filaria* infected sheep (A) and corresponding graphs showing relative transcript expression in control (C) and infected (I) lungs (B). Transcript expression is relative to the house keeping gene sheep ATPase. Boxes represent 25th and 75th quartile range, lines represents median and whiskers range of data. * Represents significant difference compared to controls.

5.4 Discussion

5.4.1 Expression of ITLN in normal sheep lungs

Previous work has shown the presence of one ITLN transcript in respiratory epithelial cells (bronchial brushings) from normal people (Kuperman et al., 2005) and two ITLN transcripts in normal mouse lung (Voehringer et al., 2007). The present study is in agreement with these findings where expression of two sITLNs, sTLN1 and sITLN3, were found to be constitutive in both the tracheal mucosa and lungs of six normal sheep. The higher transcript expression noted in the trachea may reflect the greater density of goblet cells in this tissue. Using two different antibodies, anti-ITLN peptide 1, anti-XL35 (Lee et al., 2001; French et al., 2007), ITLN was immunolocalised to goblet cells, epithelial cells, submucosal glands, glycocalyx and free mucus. Previous work has suggested that ITLN is present in epithelial cells in the intestinal tract as well as in mucus producing cells, the immunolabelling of the respiratory tract epithelial cells in this study would support this suggestion (Wrackmeyer et al., 2006). No satisfactory explanation was found for the poor/absent immunolabelling with anti-XL35 antibody in three sheep. Tissue handling, laboratory technique were kept constant and any slight difference in transcript expression did not explain the results and thus this finding was presumed to be artefactual.

The role of ITLN in the normal respiratory tract is unknown however it may have a role to play in the character of mucus by interaction with mucins. This role has been suggested by homology with the South African clawed toad, *Xenopus laevis*, egg lectin XL35, which following fertilisation, interacts with the egg jelly coat protein to induce an irreversible change that establishes a block to polyspermy (Nishihara et al., 1986; Arranz-Plaza et al., 2002). This protective layer, which is like a fluid hydrogel, is flexible enough to encapsulate the developing embryo (Arranz-Plaza et al., 2002). In the respiratory tract it has been suggested that the mucus layer acts as a matrix for protective proteins such as lysozyme and lactoferrin (Davies et al., 2002). Lactoferrin is considered to play an important role in innate defence against

respiratory tract bacteria in man (Wilmott et al., 2000; Boyton and Openshaw, 2002; Ganz, 2002).

Interestingly ITLN has also been described as a lactoferrin receptor (Suzuki et al., 2001; Suzuki and Lonnerdal, 2004; Liao et al., 2007), so rather than playing a role in the character of mucus, ITLN may be functioning as a lactoferrin receptor within the mucus. Interestingly in cystic fibrosis in man, which occurs due to a genetic mutation of the cystic fibrosis transmembrane conductance regulator (McPherson et al., 2001) and is characterised by chronic respiratory tract infections with *Pseudomonas aeruginosa* (Bisgaard et al., 1997), ITLN transcript expression (Colledge et al., 2005) and lactoferrin expression and activity are decreased (Rogan et al., 2004), possible further evidence for an antibacterial role for this molecule in the respiratory tract. Alternatively, the ability of recombinant hITLN to bind D-galactofuranosyl residues present in the arabinogalactan on the cell wall of *Nocardia rubra* (Tsuji et al., 2001) might suggest a direct role in defence against bacterial infections.

5.4.2 Expression of ITLN in a Th2 environment in sheep respiratory tract

The results of the experiments presented in this chapter are in agreement with previous publications for mouse (Kuperman et al., 2005; Voehringer et al., 2007) and humans (Kuperman et al., 2005; Wu et al., 2005) which have shown upregulation of mITLN1a and mITLN1b/2 and hITLN respectively in a Th2 environment in the respiratory tract. The findings are also in agreement with our previous study which showed that incubation of sheep tracheal explants with sIL-4 resulted in upregulation of sITLN transcript compared to medium only controls (French et al., 2007). The present work using the same model demonstrates that of the three sITLNs, sITLN2 most consistently upregulated following incubation with sIL-4, however upregulation of sITLN1 and sITLN3 occurred in some sheep. ITLN protein was also shown to upregulate in explants from the majority of sheep. The low number of explants and the variability between sheep was a limitation and a higher number of explants may have lead to statistically significant results. In the sheep respiratory cell culture

model, incubation with either sIL-4 or hIL-13 resulted in upregulation /induction of all three sITLNs. It is unfortunate that this model was only established in late 2007, and that so few replicates were available.

The upregulation of sITLN2 and sTLN3 in the lung following infection with *D. filaria* is in agreement with findings in mice where *N. brasiliensis* was shown to upregulate both mouse ITLN homologues in the lung (Voehringer et al., 2007). Interestingly overexpression of ITLN in the mouse lung did not confer any protection against *N. brasiliensis*, it remains to be shown if ITLN has a protective role to play in parasitic infections in either the respiratory or gastrointestinal tracts of other species. The significance of the upregulation of ITLN in asthmatic people (Kuperman et al., 2005) is also unknown. It may have a protective anti-bacterial role, but if it alters mucus character there is concern that it may play a role in formation of fatal asthma plugs. Interestingly lactoferrin is also increased in bronchoalveoler lavage fluid of asthmatic people and recombinant lactoferrin is currently undergoing clinical trials for the treatment of asthma (van de Graaf et al., 1991; Andersen, 2004).

5.4.3 Expression of SIAT4C in the normal respiratory tract of sheep

In the present study SIAT4C transcript expression has been shown in normal sheep trachea and lung, which is in agreement with previous findings where several SIAT4C isotranscripts have been shown in normal human trachea and lung (Grahn and Larson, 2001). SIAT4C is known to be involved in the formation of the Sialyl Lewis X (Delmotte et al., 2002) present on bronchial mucins, endothelial cells and leucocytes. It is unknown if the higher transcript expression, seen in the sheep lung compared to the trachea, is reflective of greater bronchial, endothelial or leucocyte sialylated mucin expression in the lung. The presence of acidic and neutral mucins in sheep respiratory tract is shown clearly with AB/PAS histochemistry and the acidic brush border/ glycocalyx agrees with published findings (Mariassy et al., 1988).

5.4.4 Expression of SIAT4C in a Th2 environment in the respiratory tract of sheep

SIAT4C has been shown to upregulate in human bronchial explants stimulated with the proinflammatory cytokine, TNFα (Delmotte et al., 2002) and more recently IL-1β has been shown to upregulate SIAT4C gene transcription and to directly increase expression of the Sialyl Lewis X complex in the HuH-7 hepatic carcinoma cell line (Higai et al., 2006). There are no reports on the expression of SIAT4C in a Th2 environment in the respiratory tract. The present work demonstrates the downregulation of SIAT4C in sheep tracheal explants in response to incubation with the Th2 cytokine IL-4. Respiratory tract mucins have been shown to express the Sialyl Lewis X in man, which is dependent on the expression of SIAT4C. In the present experiment, AB/PAS staining of tracheal explants showed that incubation with sIL-4 resulted in an increased number of alcian blue (acidic) goblet cells. These findings are suggestive that the change in expression of SIAT4C transcript did not appear to be specifically related to sialylation of goblet cell mucins. Further work is required to determine if this finding is repeatable in different model systems.

5.5 Conclusion

sITLNs and SIAT4C appear to be regulated differently by the Th2 cytokine IL-4 in the sheep tracheal explant model used in this study, with upregulation of sITLNs and down regulation of sSIAT4C occurring in response to incubation with this cytokine. In a natural infection with the sheep lungworm, *D. filaria*, sITLN2 and sITLN3 were shown to upregulate. It is unknown if this is a protective reponse and whether a similar reponse is seen to gastro-intestinal parasitic infections in sheep.

6 Transcript and protein expression in *Teladorsagia circumcincta* infected yearling sheep and lambs

6.1 Summary

The expression of the transcripts for sITLN1, sITLN2, sITLN3 and sSIAT4C in the abomasal mucosa were found to upregulate significantly following challenge infection with 50,000 *Teladorsagia circumcincta* L3, in yearling sheep using semi-quantitative RT-PCR, when compared to unchallenged sheep. For all three sITLNs significant upregulation was seen in challenged naïve (cnv) and challenged previously infected (cpi) yearling sheep, with upregulation occurring at an earlier time point in sheep that had been previously infected (immune) prior to challenge compared to naïve sheep. For sSIAT4C, significant upregulation was only seen in cpi yearling sheep with no significant upregulation in cnv yearling sheep compared to unchallenged groups. Challenge infection resulted in concurrent upregulation of transcripts considered typical of a Th2 response in sheep: sMCP-1, OvGal-14 and sIL-4. Similar results were found in lambs with the exception of sITLN3 where no significant upregulation was seen at any time point following challenge infection. Upregulation of ITLN protein was confirmed by Western blot and immunolocalised to abomasal mucus neck cells. The significant upregulation of sITLN transcript was confirmed in a limited number of sheep using quantitative real time RT-PCR as was the higher expression of sITLN in yearling sheep compared to lambs at day 5 following challenge infection of previously infected groups. Carbohydrate histochemistry demonstrated significantly increased neutral mucins and decreased acidic mucins in challenged naïve sheep at day 10 post challenge, compared to unchallenged naïve sheep, whilst SIAT4C transcript expression was not significantly altered at this time point.

6.2 Introduction

Previous work has shown the upregulation of ITLNs (Knight et al., 2004; Pemberton et al., 2004a; Datta et al., 2005; Artis, 2006) and SIAT4C (Knight et al., 2004; Kawai et al., 2007) in the gastro-intestinal tract of rodents infected with nematodes known to induce a Th2 response. The results of experimental work in chapters 4 & 5 has shown that three sITLNs and sSIAT4C are expressed in sheep tissues, and that furthermore upregulation of sITLNs occurs *in vitro*, ex vivo and *in vivo* in response to a Th2 stimulation in the respiratory tract of sheep whilst significant downregulation of sSIAT4C occurred in sheep tracheal explants in response to incubation with recombinant sheep IL-4. The following experimental work was undertaken to determine if sITLNs and sSIAT4C were upregulated in the gastro-intestinal of sheep in response to nematode infection as had been seen in rodents and furthermore to determine if these molecules played a role in the immune response to parasites. A challenge model of infection, with the abomasal parasite *T. circumcincta,* in naive and previously infected sheep and lambs was chosen. Previous experimental work had shown that *T. circumcincta* induced mucosal hyperplasia, eosinophil and mast cell infiltration and increased mucus production, markers of a Th2 response, and furthermore that the immune response in yearling sheep and lambs differed (Coop et al., 1985; Smith et al., 1985). This model was established at the Moredun Research Institute in 2004 by Dr. W.D. Smith and permission was obtained to collect post mortem samples from ongoing experiments 2004 - 2007. Full details of experimental design and all materials and methods are given in chapter 2. A Kolmogorov-Smirnov test was used to assess normal distribution. A Kruskal-Wallis test was used to compare all groups. If groups were significantly different a Mann-Whitney U test was used as a post hoc test for all pairwise comparisons. $P < 0.05$ was considered significant.

6.3 Results

6.3.1 Worm counts

Full details of animals and experimental protocol have been given previously (2.1.3 - 2.1.4, 2.2.5 – 2.2.8, Table 2.2). In brief, *T. circumcincta* challenge infection experiments conducted over a three year period have been grouped into four trials, numbered in chronological order, trial 1-3: yearling sheep, trial 4: lambs. For descriptive purposes, sheep were divided into the following four groups; group 1: unchallenged naïve (unv); group 2: challenged naïve (cnv) killed at set time points post challenge; group 3: unchallenged previously infected (upi) and group 4: challenged previously infected (cpi) killed at set time points post challenge. The previously infected animals were given 2000 stage 3 larvae (L3) three times per week for eight weeks. A Kruskal-Wallis test determined that the groups were significantly different ($p = 0.0001$). On post hoc analysis there were significantly lower worm counts in cpi yearling sheep at day 2, 5 and 10 post challenge infection compared to cnv at the same time points which validates the model and confirms that trickle infection in these yearling sheep has conferred a degree of immunity as previously reported (Smith et al., 1983a). In the lambs, the Kruskal-Wallis test showed groups were significantly different ($p = 0.003$) and on post hoc analysis a significantly lower worm count was present at day 10 post challenge in the cpi group compared to the cnv group at the same time point; no significant difference was present at day 5 or day 22 between these two groups. These results show that trickle infection conferred some immunity in these lambs (Figure 6.1A).

Two cross breeds of sheep were represented in trials 1-3; Dorset x Suffolk and Scottish blackface x Leicester. All trial 4 lambs were Scottish blackface x Leicester. In order to ensure there was no genetic difference in resistance to parasites between these sheep cross breeds and to ensure results were repeatable between different trials, worm counts were compared between trial 1 (2005) cnv day 10 (Scottish blackface x Leicester) and trial 2 (2006) cnv day 10 (Dorset x Suffolk), and also between trial 1 (2005) cpi day 10 (Scottish blackface x Leicester) and trial 2 (2006) cpi day 10 (Dorset x Suffolk). No significant difference was found in worm counts

between these groups of cnv and cpi sheep and thus sheep breed or trial date were not considered to affect the results and the data from trials 1-3 were combined (Figure 6.1B).

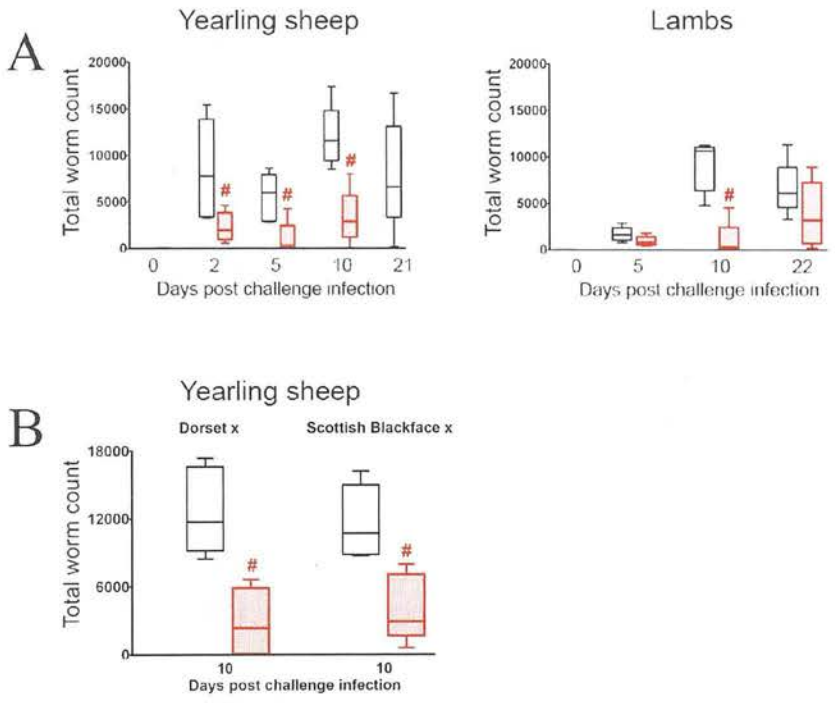


Figure 6.1: Worm counts in yearling sheep and lambs

(A) Total worm counts are shown for yearling sheep and lambs following challenge with 50,000 *T. circumcincta* L3.

(B) Total worm counts are shown for Dorset x Suffolk yearlings and Scottish blackface x Leicester yearling sheep at day 10 post challenge with 50,000 *T. circumcincta* L3.

Black boxes represent challenged naïve (cnv) sheep, red boxes represent challenged previously infected (cpi) sheep. # represents significant difference between cnv and cpi groups. Boxes represent 25th and 75th quartiles, line within box the median and whiskers the range of data.

6.3.2 Transcript expression in abomasal mucosa of *T. circumcincta* infected yearling sheep

6.3.2.1 Introduction

Abomasal mucosal tissue was collected (2.2.8) from the following groups of yearling sheep; unchallenged naïve (unv) (n = 6), unchallenged previously infected (upi) (n = 6), challenged naïve (cnv); days 2 (n = 6), 5 (n = 6), 10 (n = 12), 21 (n = 9) and challenged previously infected (cpi); days 2 (n = 6), 5 (n = 6), 10 (n = 12), in total n = 69. Samples were from yearling sheep in trials 1, 2 and 3 as detailed in Table 2.2 with the exception of trial 3 unchallenged naïve sheep, which were not included. Data from all three trials was combined. In order to demonstrate evidence of a Th2 response in the abomasal mucosa, RNA was extracted and semi-quantitative RT-PCR was used to determine expression of transcripts for genes considered markers of a Th2 response; sheep mast cell protease-1 (sMCP-1), ovine galectin-14 (OvGal-14) and sIL-4. The expression of transcripts of the genes of interest sITLN1, sITLN2, sITLN3 and sSIAT4C were then assessed in this model. For all transcripts, expression was determined relative to the housekeeping gene sheep ATPase. Full details of molecular biology techniques used have been described previously (2.3.1.2, 2.3.2 – 2.3.8, 2.4.3, Table 2.6).

6.3.2.2 Expression of sIL-4, sMCP-1 and OvGal-14 transcripts in abomasal mucosa of T. circumcincta infected yearling sheep

There was significant upregulation of expression of sIL-4 and OvGal-14 transcripts in the cpi compared to the upi at all time points (days 2, 5 and 10) whilst significant upregulation was only seen at days 10 and 21 when cnv sheep were compared to unv sheep. Transcript expression was significantly higher in the cpi compared to the cnv at all time points (days 2, 5 and 10) for sIL-4 and at day 5 for OvGal-14. Expression of sMCP-1 was significantly higher in upi compared to unv and in cpi compared to cnv at days 2 and 5. Significantly higher expression was seen in cnv sheep at days 2, 10 and 21 compared to unv sheep whilst no increase in expression was seen in cpi sheep compared to upi sheep (Figure 6.2, Table 6.1).

6.3.2.3 Expression of sITLN transcripts in abomasal mucosa of T. circumcincta infected yearling sheep using semi-quantitative RT-PCR

There was significantly higher expression of all three sITLN transcripts at day 10 in the cpi when compared to the upi sheep. This was the only time point when sITLN3 was found to be significantly upregulated. When cnv sheep were compared to unv significant upregulation of sITLN 1 transcript was found at days 10 and and sITLN 1 and 2 at day 21 whilst when cpi were compared to upi sheep significant upregulation was found at days 2, 5 and 10 for sITLN1 and days 5 and 10 for sITLN2. At day 5 post challenge, sITLN 1 and 2 transcript relative expression were found to be significantly higher in cpi compared to the cnv sheep (Figure 6.2, Table 6.1).

6.3.2.4 Expression of sSIAT4C transcript in abomasal mucosa of T. circumcincta infected yearling sheep using semi-quantitative RT-PCR

There was significantly higher expression of SIAT4C transcript in the cpi yearling sheep at days 5 and 10 compared to the upi yearling sheep. On the other hand when the cnv were compared to the unv sheep they were either significantly down regulated (day 2) or showed no significant difference in transcript expression (days 5, 10, 21). The unv sheep had significantly higher transcript expression than the upi sheep (Figure 6.2, Table 6.1).

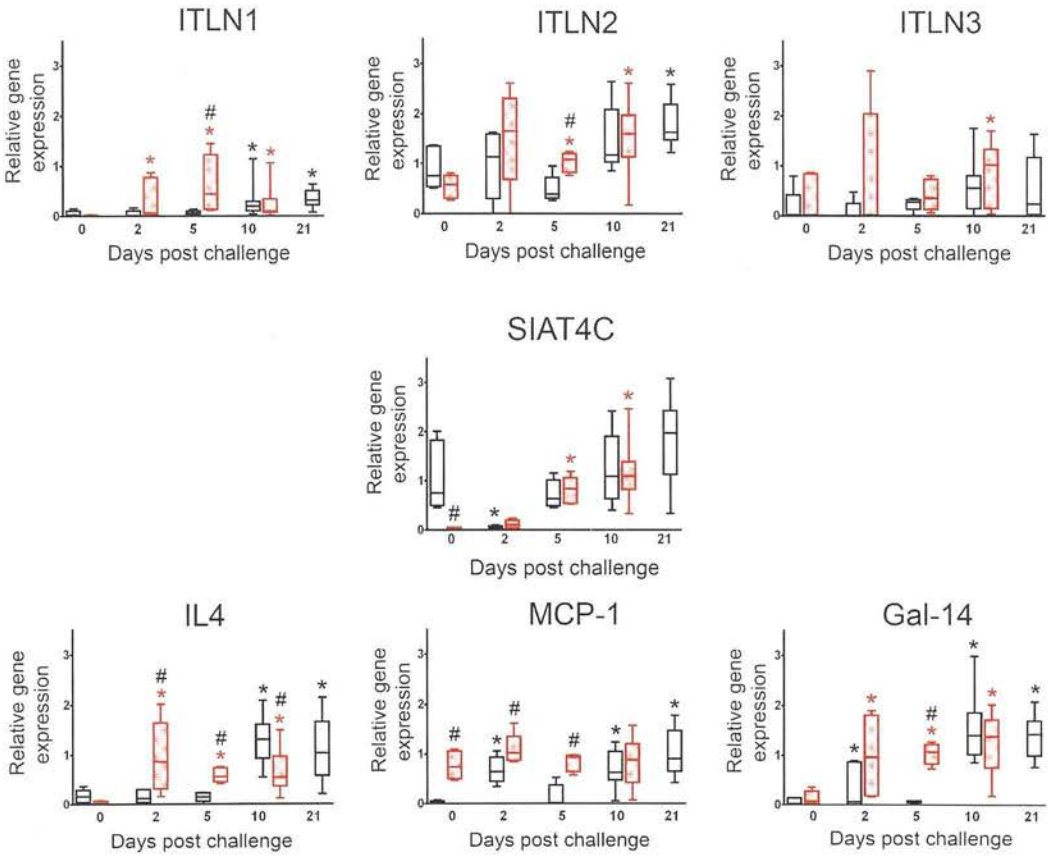


Figure 6.2: Expression of transcripts in yearling sheep – semi quantitative RT-PCR

Box and whisker plots showing relative gene transcript expression by RT-PCR in abomasal mucosa of unchallenged naïve (unv), unchallenged previously infected (upi), *T. circumcincta* challenged naïve (cnv) and *T. circumcincta* challenged previously infected (cpi) sheep. Expression is relative to the house keeping gene, sheep ATPase. Semi-quantitative RT-PCR was carried out as described in Materials and Methods. The naïve groups are shown as clear black boxes, whilst the previously infected groups as red hatched boxes. Boxes represent the 25th and 75th quartiles, median is shown within the box, the whiskers represent the range of the data. Numbers (2, 5, 10, 21) represent days post challenge, the upi and unv groups are labelled 0 for day 0. Asterix represents significant difference between cnv and unv (*), cpi and upi (*), whilst hatch (#) represents significant difference between unv and upi or cnv and cpi at the same time points post challenge. A Kruskal-Wallis test

was used to compare groups for each transcript of interest and a Mann-Whitney U as a post hoc test for pairwise comparisons. $P < 0.05$ was considered significant.

	Gal- 14	sIL-4	sMCP-1	sITLN1	sITLN2	sITLN3	sSIAT4C
Kruskal-Wallis	**<0.0001**	**<0.0001**	**0.0001**	**<0.0001**	**0.0001**	**0.0398**	**<0.0001**
unv v 2cnv	0.5887	0.8182	**0.0022**	0.5887	0.4848	0.0931	**0.0022**
unv v 5cnv	0.3939	0.9372	0.5887	0.5887	0.0931	0.3095	0.5887
unv v 10cnv	**0.0009**	**0.0009**	**0.0012**	**0.0043**	0.0441	0.1223	0.426
unv v 21cnv	**0.0004**	**0.0028**	**0.0004**	**0.0008**	**0.0016**	0.1447	0.0879
upi v 2cpi	**0.0411**	**0.0022**	0.132	**0.0043**	0.0649	0.999	0.0649
upi v 5cpi	**0.0022**	**0.0022**	0.8182	**0.0022**	**0.0043**	0.3939	**0.0022**
upi v 10cpi	**0.0017**	**0.0009**	0.8149	**0.0017**	**0.0057**	**0.017**	**0.0009**
unv v upi	0.3095	0.0931	**0.0022**	0.1797	0.1797	0.9372	**0.0022**
2cnv v 2cpi	0.0649	**0.0087**	**0.0152**	0.0649	0.3095	0.9372	0.0931
5cnv v 5cpi	**0.0022**	**0.0022**	**0.0022**	**0.0043**	**0.0087**	0.6991	0.3939
10cnv v 10cpi	0.3408	**0.0051**	0.5068	0.5834	0.977	0.3124	0.977

Table 6.1: Statistical results for semi-quantitative RT-PCR in yearling sheep
Comparison of relative expression of transcripts in challenged naïve (cnv) and unchallenged naïve (unv), challenged previously infected (cpi) and unchallenged previously infected (upi), and previously infected and naïve yearling sheep. Numbers (2, 5, 10, 21) represent days post challenge with 50,000 *T. circumcincta* L3. Bold type represents statistically significant difference. Gene expression is relative the housekeeping gene sheep ATPase. A Mann-Whitney U was used as a post hoc test for pairwise comparisons.

6.3.3 Transcript expression in abomasal mucosa of *T. circumcincta* infected lambs

6.3.3.1 Introduction

Abomasal mucosal tissue was collected (2.2.8) from the following groups of lambs; unchallenged naïve (unv) (n = 4), challenged naïve (cnv); days 5 (n = 6), 10 (n = 5), 22 (n = 4) and challenged previously infected (cpi); days 5 (n = 6), 10 (n = 5), 22 (n = 5) in total n = 35. Samples were all from trial 4, Scottish blackface x Leicester

lambs as detailed in Table 2.2. As for the yearling sheep, in order to demonstrate evidence of a Th2 response in the abomasal mucosa, RNA was extracted and semi-quantitative RT-PCR was used to determine expression of transcripts for genes considered markers of a Th2 response; sheep mast cell protease-1 (sMCP-1), ovine galectin-14 (OvGal-14) and sIL-4. The expression of transcripts of the genes of interest sITLN1, sITLN2, sITLN3 and sSIAT4C were then assessed. For all transcripts expression was determined relative to the housekeeping gene sheep ATPase. There was no upi group in this trial and challenged groups (previously infected and naïve) are compared to the unv group. Full details of molecular biology techniques used have been described previously (2.3.1.2, 2.3.2 – 2.3.8, 2.4.3, Table 2.6)

6.3.3.2 Expression of sIL-4, sMCP-1 and OvGal-14 transcripts in abomasal mucosa of T. circumcincta infected lambs using semi-quantitative RT-PCR

For sMCP-1 significantly higher transcript expression was seen at day 10 and 22 in cpi compared to unv whilst there was no significant upregulation in cnv compared to unv lambs at any time point. At day 10 sMCP1 transcript expression was significantly higher in cpi compared to cnv lambs at the same time point. The Kruskal-Wallis test showed no significant difference in transcript expression between groups for sIL-4 and for Ovgal-14 and thus no post hoc test was applied. (Figure 6.4, Table 6.2).

6.3.3.3 Expression of sITLN transcripts in abomasal mucosa of T. circumcincta infected lambs using semi-quantitative RT-PCR

There was significantly higher transcript expression of sITLN1 transcript in cpi lambs compared to unv at all time points (days 5, 10 and 22) whilst significantly higher expression was only seen at day 22 when cnv were compared to unv. For sITLN2 significantly higher transcript expression was seen at day 5 when cpi were compared to cnv and at days 10 and 22 when cnv were compared to unv. There was significantly higher expression of transcript in the cpi compared to the cnv sheep at day 5 for sITLNs 1 and 2 and also at day 22 for sITLN2. For sITLN3 no significant

difference in expression was shown between groups and thus no post hoc test was applied (Figure 6.4 and Table 6.2). Ethidium bromide gels of sITLN1, 2 and 3 transcript expression in lambs and yearling sheep are shown in Figure 3.

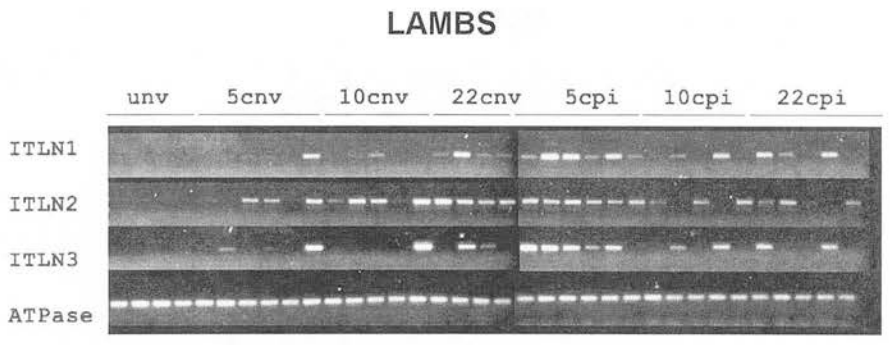


Figure 6.3: Ethidium bromide gels showing expression of sITLN transcripts in lambs

Ethidium bromide gel (1.4%) of sheep intelectin (sITLN) 1, 2 and 3 transcripts in lambs from Trial 4; unchallenged naïve (unv), challenged naïve (cnv) (days 5, 10, 22) and challenged previously infected (cpi) (days 5, 10 and 22). Expression of the housekeeping gene sheep ATPase is shown for comparison. Groups numbers were as follows: unv, 22cnv (n = 4), groups 10cnv, 10cpi, 22cpi (n = 5) and groups 5cnv, 5cpi (n = 6).

6.3.3.4 Expression of sSIAT4C transcript in abomasal mucosa of T. circumcincta infected lambs using semi-quantitative RT-PCR

There was significantly higher expression of sSIAT4C transcript at all time points when cpi were compared to unv whilst cnv groups did not show significant higher expression at any time point when compared to unv animals. At day 5 there was significantly higher transcript expression in cpi lambs compared to cnv at the same time point (Figure 6.4, Table 6.2).

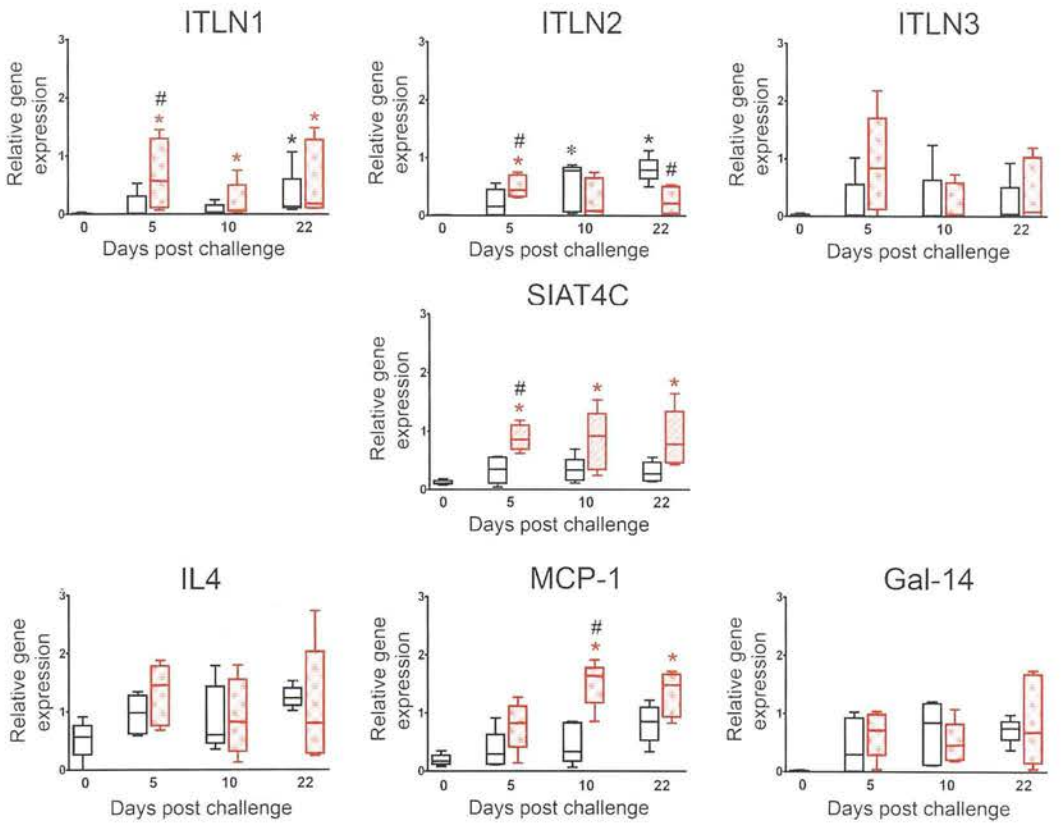


Figure 6.4: Expression of transcripts in lambs - semi quantitative RT-PCR

Box and whisker plots showing relative gene transcript expression by RT-PCR in abomasal mucosa of unchallenged naïve (unv), *T. circumcincta* challenged naïve (cnv) and *T. circumcincta* challenged previously infected (cpi) sheep. No unchallenged previously infected group was present in this experiment. The house keeping gene was sheep ATPase. Semi-quantitative RT-PCR was carried out as described in Materials and Methods. The naïve groups are shown as clear black boxes, whilst the previously infected groups as red hatched boxes. Boxes represent the 25th and 75th quartiles, median is shown within the box, whiskers represent the range of the data. Numbers (5, 10, 22) represent days post challenge, the unv group is labelled 0 for day 0. Asterix represents significant difference between cnv (*) or cpi (*) and unv group, whilst hatch (#) represents significant difference between cnv and cpi groups at the same time points post challenge. A Kruskal-Wallis test was used to compare groups for each transcript of interest and a Mann-Whitney U as a post hoc test for pairwise comparisons. $P < 0.05$ was considered significant.

	Gal 14	IL-4	sMCP1	ITLN1	ITLN2	ITLN3	SIAT4C
Kruskal-Wallis	0.0749	0.2865	**0.0018**	**0.0041**	**0.01**	0.6075	**0.002**
unv v 5cnv	n/a	n/a	0.6095	0.1714	0.0667	n/a	0.1714
unv v 10cnv	n/a	n/a	0.4127	0.0635	**0.0159**	n/a	0.0635
unv v 22cnv	n/a	n/a	0.0571	**0.0286**	**0.0286**	n/a	0.1143
unv v 5cpi	n/a	n/a	0.0667	**0.0095**	**0.0095**	n/a	**0.0095**
unv v 10cpi	n.a	n.a	**0.0159**	**0.0317**	0.2857	n.a	**0.0159**
unv v 22cpi	n/a	n/a	**0.0159**	**0.0159**	0.0635	n/a	**0.0159**
5cnv v 5cpi	n/a	n/a	0.0931	**0.026**	**0.0411**	n/a	**0.0022**
10cnv v 10cpi	n/a	n/a	**0.0159**	0.2222	0.2508	n/a	0.0952
22cnv v 22cpi	n/a	n/a	0.1111	0.5556	**0.0317**	n/a	0.0635

Table 6.2: Statistical results for semi-quantitative RT-PCR in lambs

Comparison of relative transcript expression in challenged naïve (cnv) and unchallenged naïve (unv), challenged previously infected (cpi) and unv, and previously infected and naïve yearling sheep at different time points. Numbers (2, 5, 10, 21) represent days post challenge with 50,000 *T. circumcincta* L3. Bold type represents statistically significant difference. A Mann-Whitney U was used as a post hoc test for pairwise comparisons. No post hoc test was undertaken if the Kruskal-Wallis test showed no significant difference between groups. Not applicable (n/a).

6.3.4 Expression of sITLN transcript in abomasal mucosa of *T. circumcincta* infected yearling sheep and lambs using real time quantitative RT-PCR

6.3.4.1 Introduction

In the previous experiment (6.3.3) using semi-quantitative RT-PCR expression of sITLN2 transcript was shown to be increased at day 5 in the cpi compared to the cnv yearling sheep. Moreover, the relative expression of sITLN2 transcript appeared to be higher in the yearling sheep compared to the lambs at this time point (6.3.3, 6.3.4). In order to confirm these findings, quantitative RT-PCR was undertaken using

four groups of sheep; day 5 cpi yearling sheep, day 5 cnv yearling sheep, unv yearling sheep and day 5 cpi lambs. The number of groups was limited to 4 as this was the maximum number that could be run at the one time on the Opticon 1 real-time PCR machine (MJ Research, GRI, Rayne, UK) where it is advised to run samples in triplicate on a 96 reaction plate with triplicate copies of standards and multiple blanks. Full details of methodology are given in chapter 2 (2.3.1.2, 2.3.2, 2.3.4 – 2.3.5, 2.3.9). The primers used were not specific and identified sITLN.

6.3.4.2 Expression of sITLN transcript in abomasal mucosa of T. circumcincta infected yearling sheep and lambs using real time quantitative RT-PCR

There was no significant difference in sITLN transcript expression when day 5 cnv yearling sheep were compared to unv yearling sheep (p = 0.5887). Significantly higher transcript expression was shown when the following groups were compared: day 5 cpi yearling sheep compared to unv yearling sheep (p = 0.0087), data not shown; day 5 cpi yearling sheep compared to day 5 cnv yearling sheep (p = 0.0022) and day 5 cpi yearling sheep compared to day 5 cpi lambs (p = 0.0087), (Figure 6.5).

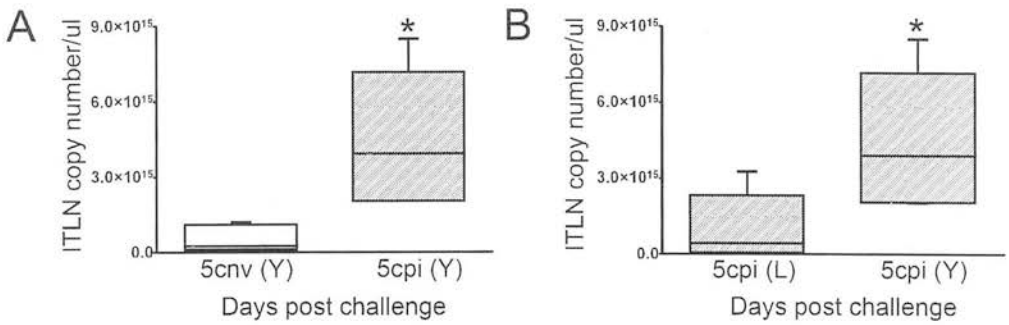


Figure 6.5: Quantitative RT-PCR for ITLN transcript expression in selected yearling sheep and lambs

Box and whisker plots showing (A) ITLN copy number in abomasal mucosa of *T. circumcincta* challenged naïve (cnv) and previously infected (cpi) yearling sheep (Y) day 5 post challenge with 50,000 L3 and (B) *T. circumcincta* challenged previously infected (cpi) yearling sheep and lambs (L) day 5 post challenge with 50,000 L3.

Quantitative RT-PCR was carried out as described in Materials and Methods. The naïve groups are shown as clear black boxes, whilst the previously infected groups as shaded boxes. Boxes represent the 25th and 75th quartiles, median is shown within the box, whiskers represent the range of the data. Asterix represents significant difference between groups.

6.3.5 ITLN protein expression in *T. circumcincta* infected yearling sheep

6.3.5.1 Introduction

The expression of three different ITLN transcripts had been shown in abomasal mucosa of yearling sheep and moreover significantly higher expression had been demonstrated in response to challenge with 50,000 *T. circumcincta* L3 larvae with all three sITLNs at day 10 in cpi compared to upi yearling sheep. Western blot was used to confirm protein expression in abomasal tissue and immunohistochemistry to localise expression. The peptide to which the affinity purified chicken peptide 1 antibody was raised (Figure 4.1) is present in all three sheep ITLNs and thus the antibody is non selective and does not discriminate between the three sheep ITLNs.

6.3.5.2 Western blot of abomasal mucosa to demonstrate ITLN expression

Full details of method have been given previously in chapter 2 (2.5.2). In brief, protein was extracted from abomasal mucosal samples from trial 2 sheep (n=36) using a urea extraction buffer. To allow loading of all samples, two SDS-PAGE mini gels (Criterion, Bio-Rad, California, USA) were used which allowed loading of 20 samples/gel. Equal protein loading of gels was confirmed using Imperial stain, gels were blotted and membranes incubated with affinity purified antibody to ITLN peptide 1 and beta actin as a house keeping control. Chemiluminescence was used to visualise ITLN and beta actin protein.

Low levels of protein expression were seen in three of the six unv sheep, whilst expression was not detected in the remaining three. Low levels of protein expression

were detected in five of the six cnv sheep at day 5 following challenge whilst strong protein expression was present in all the cpi sheep at the same time point. Protein expression was seen in all cnv sheep at day 10, and day 21 and in cpi sheep at day 10 post challenge. Beta actin protein expression was found to be relatively consistent in all samples with slightly lower expression seen in some of the unv sheep (Figure 6.6).

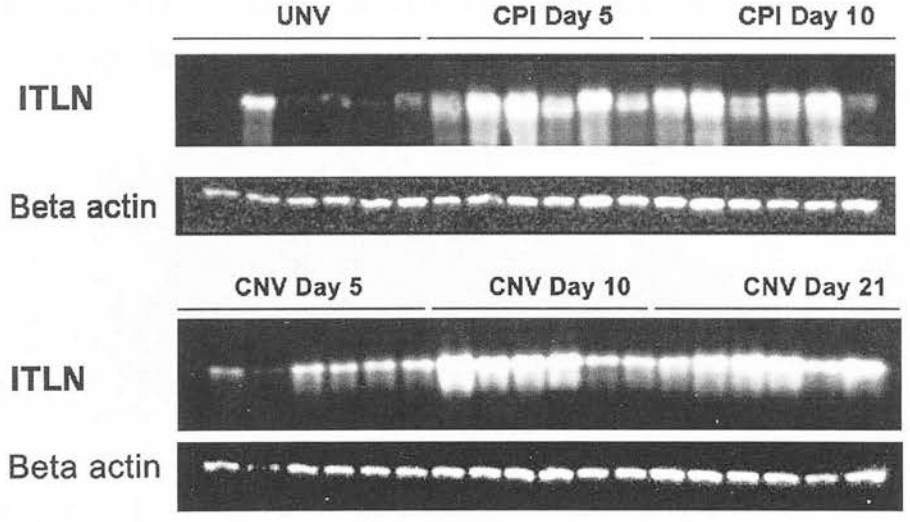


Figure 6.6: Western blot showing ITLN protein expression in yearling sheep
Western blot showing expression of the 38kD sheep ITLN in abomasal mucosa of unchallenged naïve sheep (unv), challenged previously infected sheep (cpi) at days 5 and 10 post challenge with 50,000 *T. circumcincta* L3 and challenged naïve (cnv) sheep at days 5, 10 and 21 post challenge with 50,000 *T. circumcincta* L3. For each group n = 6. Expression of beta actin is shown as a control. Protein loading was equal (not shown).

6.3.5.3 Immunohistochemistry of abomasal mucosa to demonstrate ITLN expression

The immunohistochemistry technique was optimised using sheep respiratory tissue. The affinity purified antibody to ITLN peptide1 (5 µg/ml) was selected for use on abomasal mucosa of trial 1 and 2 yearling sheep using an antigen retrieval step with Tris-HCl/EDTA/Tween 80 pH 9 as the buffer. A sequenza was not used in order to

improve quality of staining. Nova Red (Vector Laboratories) was used as the chromogen and slides were counterstained with hematoxylin. Full details of the immunohistochemistry technique are given in chapter 2 (2.6.1 – 2.6.2).

ITLN protein expression was immunolocalised to abomasal mucus neck cells (Figure 6.7A and B) and staining was higher in intensity in cnv and cpi sheep at 10 days post challenge with 50, 000 *T. circumcincta* L3 compared to unv sheep (Figure 6.7). It was notable that ITLN was detected in the gastric mucus surrounding the developing larvae and within the intestine of the parasite (Figure 6.7C).

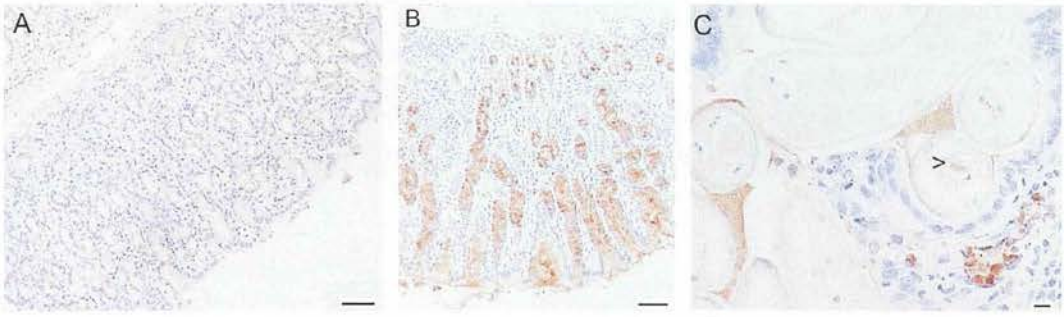


Figure 6.7: ITLN immunohistochemistry of abomasal mucosa

Immunoperoxidase labelling of sections with affinity purified chicken anti-ITLN peptide I (anti-ITLN peptide) was carried out as described in chapter 2. Sections are from an unchallenged naïve sheep (A), a challenged previously infected sheep 10 days post challenge with 50,000 *T. circumcincta* L3 (B), and a challenged naïve sheep 10 days post challenge with *T. circumcincta* demonstrating ITLN positive mucus surrounding an L4 larva with positive staining of mucus in the lumen of the larval gut (C, arrowed). Control IgY labelling was negative (not shown). Bars represent 50 μm (A, B) and 10 μm (C).

6.3.6 Carbohydrate histochemistry of abomasal mucosa

Sections from unv yearling sheep (MRI trial 2, n = 6), cnv (MRI trial 1, n = 6) and cpi yearling sheep day 10 post challenge (MRI trial 1, n = 6) were labelled with Alcian blue/Periodic acid Schiff, which labels acidic mucins torquoise and neutral mucins magenta. Sections were given a grade 0, 1, 2 or 3 blinded to the identity of

the section, for Alcian blue (AB) and for Periodic acid Schiff (PAS) staining. A Kruskal-Wallis test demonstrated significant difference between groups; PAS (p = 0.004), AB (p = 0.0022). The Mann-Whitney U was used as a post hoc test. There was significantly greater PAS staining in cnv compared to unv (p = 0.0022) and cnv compared to cpi (p = 0.0411) yearling sheep. There was significantly less AB staining in cnv compared to unv yearling sheep (p = 0.0087). (Figure 6.8)

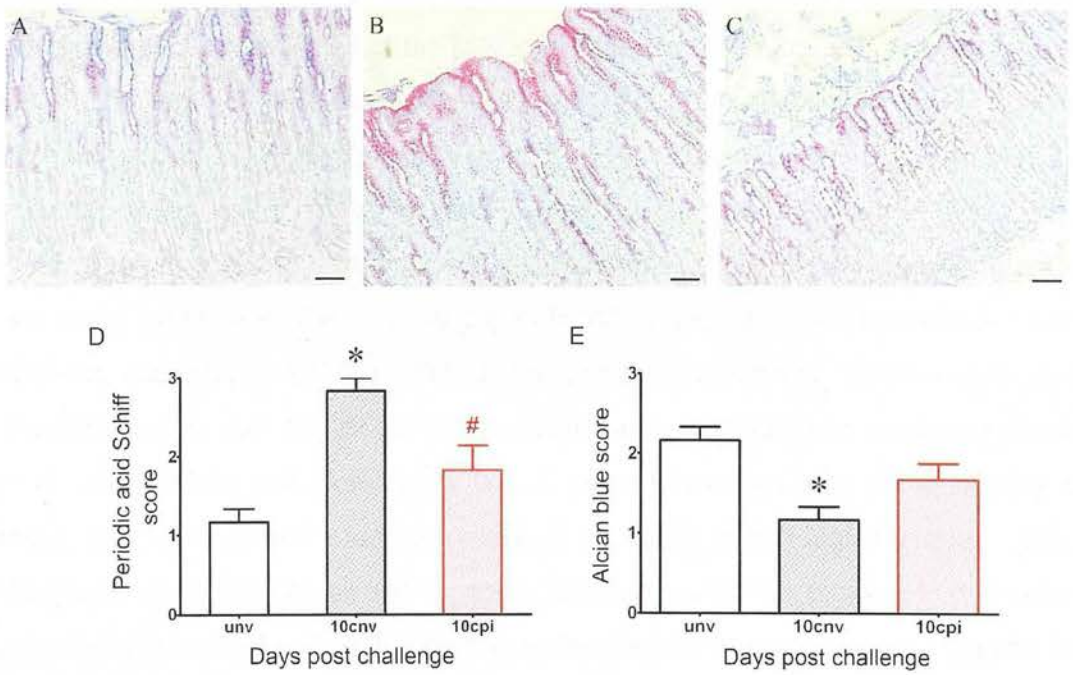


Figure 6.8: Carbohydrate histochemistry of sheep abomasal mucosa

Alcian blue/periodic acid Schiff (AB/PAS) staining of abomasal mucosa sections of (A) unchallenged naïve yearling sheep (unv), (B) challenged naïve (cnv), and (C) challenged previously infected (cpi) yearling sheep 10 days post challenge with 50,000 *T. circumcincta* L3. Bars (A, B, C) represent 50 μm.

Graphs of periodic acid Schiff (D) and alcian blue (E) scoring of abomasal mucosa sections from unv (n = 6), cnv (n = 6) and cpi (n = 6) yearling sheep, 10 days post challenge with 50,000 *T. circumcincta* L3. Bars represent mean and SEM. Asterix (*) represents significant difference between cnv and unv group, hatch (#) represents significant difference between cpi and cnv groups. A Kruskal-Wallis test was used

to compare groups and a Mann-Whitney U as a post hoc test. $P < 0.05$ was considered significant.

6.4 Discussion

6.4.1 *T. circumcincta* infection model

The present study examines the expression of sITLNs and sSIAT4C in sheep abomasal mucosa in response to *T. circumcincta* infection using a previously published challenge infection model of naïve and trickle infected (immune) yearling sheep and lambs established at the Moredun Research Institute (Smith et al., 1983b, a). ITLNs and SIAT4C, are mucus associated molecules (Pemberton et al., 2004a; Wrackmeyer et al., 2006; Yamauchi et al., 2006), which may have a role to play as effector molecules in gastrointestinal parasitic infections (Knight et al., 2004; Pemberton et al., 2004a; Datta et al., 2005; Nair et al., 2006). Parastic gastroenteritis is a major problem in sheep farming worldwide causing significant production loss (Newton and Meeusen, 2003). Whilst the genera *Haemonchus, Teladorsagia* and *Trichostrongylus* spp. can all contribute significantly to this disease syndrome (Scott et al., 1998; Miller and Horohov, 2006), *T. circumcincta* has been shown to play a major role in temperate climates (Craig et al., 2006; Smith and Zarlenga, 2006; Wrigley et al., 2006; Wilson and Sargison, 2007). A worldwide increase in resistance to anthelmintics has resulted in a need to understand the immune response in order to identify novel treatment/control strategies (Hughes et al., 2007; Traversa et al., 2007).

In a natural infection with *T. circumcincta*, infective L3 develop on the pasture and are ingested in small numbers repeatedly as animals graze. Once ingested they exsheathe in the rumen and develop into fourth and fifth stage larva (L5) in the abomasal glands in about 10 days. The L5 then mature into adult worms on the abomasal mucosa after approximately 18 days. Previous studies have shown that immunity can develop on repeated exposure to *T. circumcincta* (Smith, 1983a; Smith, 1983b) and the results of the present experiments are in agreement where lower worm counts were found in cpi sheep compared to cnv sheep. Interestingly in the present experiments, lambs were also shown to develop a degree of immunity

following trickle infection, contrary to previous studies (Smith et al., 1985). It is unknown if this difference in results could be due to genetic factors, which have previously been shown to influence immunity (Davies et al., 2006; Stear et al., 2007a). The trickle infection rate as well as the challenge dose of L3 can alter the outcome of infection (Hong et al., 1986, 1987), and thus it is important to be aware, that any model system, is not a perfect replicate of what occurs naturally.

6.4.2 *T. circumcincta* and a Th2 response

Infection with *T. circumcincta* results in abomasal mucosal mast cell and eosinophil infiltration and increased mucus production (Stevenson et al., 1994; Barrett et al., 1998; Scott et al., 1998; Scott et al., 2000; Simpson, 2000), all markers of a Th2 type response. Using published genetic markers of mast cells (sMCP-1) (Pemberton et al., 2000) and eosinophils (OvGal-14) (Dunphy et al., 2002), the results of the present study are in agreement with previous pathological findings, with evidence of increased sMCP-1 and OvGal-14 transcript expression in the abomasal mucosa following challenge infection of yearling sheep compared to unchallenged sheep. There was evidence of concurrent increased sIL-4 transcript expression in the abomasal mucosa following challenge, further evidence of a Th2 reponse. The recently published cytokine response, in the gastric lymph node of the yearling sheep used in the present experiment, confirms a strong Th2 type response to *T. circumcincta* (Craig et al., 2007). Interestingly, in the same study, the pro-inflammatory cytokines IL1β, TNFα and IL-6 were also shown to upregulate in the gastric lymph node, evidence of a concurrent pro-inflammatory response (Craig et al., 2007).

Whilst cpi lambs mounted an immune response and showed lower worms counts than cnv lambs in the present experiment, it is interesting that some marked differences were noted in transcript expression between yearling sheep and lambs. In yearling sheep, IL-4 transcript was significantly higher in cpi yearlings compared to cnv yearlings at days 2, 5 and 10 post challenge whilst no significant difference was seen between groups for lambs. For sMCP-1 signficant upregulation was seen in cnv

compared to unv yearling sheep (days 2, 10 and 21) whilst no significant difference was seen in lambs. Previous work has suggested that the maturing immune system results in different immune responses to parasites in yearling sheep and lambs and the present results would support this concept (Kambara and McFarlane, 1996; Vervelde et al., 2001).

OvGal-14 transcript expression peaked at day 10 post-challenge in cnv yearling sheep in agreement with previous work where peripheral eosinophilia was shown to peak 8 -10 days post-challenge infection in naïve sheep (Henderson and Stear, 2006). Whilst eosinophils have been associated with killing incoming larvae of *Haemonchus contortus* (Balic et al., 2006), no protective role has been shown to date in *T. circumcincta* infection. It is of interest that adult stages of *T. circumcincta* and excretory/secretory material from *T. circumcincta* L3s have been shown to produce potent chemo-attractant activity for bone marrow-derived eosinophils *in vitro* raising the question whether eosinophils may be permissive towards, rather than protective against, the parasites (Wildblood et al., 2005). In the present experiment, the upregulation of OvGal-14 transcript at an earlier time point in cpi yearling sheep compared with cnv yearling sheep may support a protective role for eosinophils.

Of particular interest was the very high level of sMCP-1 transcript in upi compared with unv yearling sheep which may support a role in immunity. It is unfortunate that an upi group was not available for lambs to see if a similar response was present in lambs. Previous work has shown an association between increased numbers of degranulated mast cells (globule leukocytes) in the mucosa and fewer nematodes in sheep (Seaton et al., 1989; Nginyi et al., 2001) and mast cells have been implicated in a hypersensitivity reaction in immune sheep resulting in rapid expulsion of gastro-intestinal parasites (Miller, 1996; Nginyi et al., 2001).

6.4.3 *T. circumcincta* and ITLNs

Infection with *T. circumcincta* is characterised by mucosal hyperplasia. A considerable number of studies have focused on the role of immunoglobulins ((Stear

et al., 2004; Martinez-Valladares et al., 2005; Halliday et al., 2007)Martinez-Valladares, 2005; Halliday, 2007; Stear, 2004), mast cells (Huntley et al., 1995) and eosinophils (Wildblood et al., 2005; Henderson and Stear, 2006), in natural immunity, however, very little work has focused on the role of mucus and the constituents of mucus. ITLN has been immunolocalised to mucus producing cells in the gastrointestinal tract of rodents (Pemberton et al., 2004a; Artis, 2006) and results from chapter 4 suggest that ITLN is also expressed in mucus producing cells in sheep. In the present study, upregulation of ITLN protein has been shown in the abomasal mucosa of cnv and cpi yearling sheep compared to unv sheep by Western blot. The expression of ITLN protein in five of six sheep as early as day 5 post-challenge of naïve animals is supportive of a role for ITLN in the early response to parasites. Immunohistochemistry has demonstrated increased intensity of ITLN staining in mucous neck cells of *T. circumcincta* challenged sheep compared to unv yearling sheep and furthermore ITLN positive mucus was shown surrounding *T. circumcincta* larvae which might suggest a role for ITLN in entrapment of parasites. The anti-ITLN peptide 1 antibody used for Western blot and immunohistochemistry does not discriminate between different sITLNs and transcript expression was examined in the abomasal mucosa for the three sITLNS. The significant upregulation of the three sITLN transcripts in cpi yearling sheep compared to upi groups is in agreement with findings in rodent models (Pemberton et al., 2004a; Datta et al., 2005; Artis, 2006; Voehringer et al., 2007). Interestingly sITLN1 and sITLN2 also showed significant upregulation in cnv compared to unv yearling groups. The earlier upregulation of sITLN1 and sITLN2 in cpi yearling sheep compared to cnv yearling sheep would support a role for ITLN in the immune response to *T. circumcincta.*

The expression of sITLN transcripts was also examined in *T. circumcincta* infected lambs and interestingly results were very comparable to yearling sheep with significant upregulation of sITLN1 and sITLN2 transcripts occurring in cpi and cnv lambs compared to unv lambs. Expression of sITLN1, 2 and 3 transcripts was absent in unv lambs and induced as early as day 5 post challenge in some lambs, however, because of the marked variation in expression between lambs following challenge, this was not found to be statistically significant. A further interesting observation was

the presence of higher expression of sITLN2 in cpi yearlings at day 5 post challenge compared to lambs at the same time point. A finding that was confirmed by quantitative RT-PCR. Further evidence of an age related difference in immune response. Overall, these findings are supportive for a role for ITLN in the early response to *T. circumcincta* and also as part of the acquired immune response.

ITLN may play a role in altering the characteristics of mucus leading to worm entrapment. Previous studies have shown that on fertilisation the frog egg lectin (XL35; 60% identity with mammalian ITLNs) interacts with mucin-like proteins to alter the characteristics of the egg coat protein and thus prevents polyspermy (Chang et al., 2004). Recombinant hITLN1 has been shown to recognise D-galactofuranosyl residues present in the arabinogalactan on the cell wall of *Nocardia rubra* (Tsuji et al., 2001) and thus ITLN may play a protective antibacterial role following mucosal damage by invading parasites. It is of interest that the homologous ascidian plasma lectin has been shown to increase phagocytic activity of *Halocynthia roretzi* hemocytes towards sheep red blood cells (Abe et al., 1999) and may support a role for ITLN in cell activation. In a transgenic mouse model, mITLN1a and mITLN1b/2 have recently been shown to be part of the innate response to *Nippostrongylus braziliensis* infection. However over-expression of ITLN in the lung had no effect on parasite expulsion (Voehringer et al., 2007). Thus, it is uncertain if ITLN does have a protective role in nematode infections.

It is of interest that another lectin, ovine galectin-11, subsequently renamed galectin-15 (Gray et al., 2004), has been found in abundance in gastro-intestinal tract mucus following helminth infection and a role in altering the properties and activities of immune mucus has been suggested (Dunphy et al., 2000). In the highly glycosylated brush border/mucus interface, secreted lectins such as galectin-15 and ITLNs are ideally located to influence mucus properties.

6.4.4 *T. circumcincta* and SIAT4C

Transient alteration of intestinal mucin sialylation has been reported in *N. brasiliensis* infection in rats (Karlsson et al., 2000), changes which were thought to be secondary to alterations in the expression of CMP-NeuAC hydroxylase and GalNac transferase (Karlsson et al., 2000). Upregulation of the sialyltransferase, SIAT4C, has been shown in the jejunum of *T. spiralis* infected mice ((Knight et al., 2004) and *N. brasiliensis* infected rats (Yamauchi et al., 2006; Kawai et al., 2007). In the present *T. circumcincta* study, as reported in the rodent studies, SIAT4C transcript was shown to significantly upregulate in cpi yearling sheep compared to upi yearling sheep at days 5 and 10 post challenge with 50,000 *T. circumcincta* L3, whilst no significant change in expression was seen in cnv compared to unv sheep. Results in lambs were similar, with significantly higher transcript expression in cpi lambs compared to unv lambs whilst no significant difference was seen between cnv and unv lambs. These findings would support a role for SIAT4C in the immune response to *T. circumcincta*. Upregulation of SIAT4C has been shown in euthymic and athymic *N. brasiliensis* infected rats, suggestive that SIAT4C may be regulated by both thymus dependent and independent mechanisms (Kawai et al., 2007) and interestingly, higher expression of SIAT4C transcript was seen in the euthymic rats.

It is possible, that the glycosylation status of the terminal sugar chains of mucins may have a role to play in rejection of parasites. Unfortunately, no antibody was available for SIAT4C and immunolocalisation was not possible. Thus, without immunolocalisation one cannot be sure that any upregulation of SIAT4C transcript expression observed in the present work, was mucin related. In fact, both activated T and B lymphocytes have been shown to express SIAT4C (Blander et al., 1999). Furthermor, SIAT4C is responsible for the sialylation of the Sialyl Lewis X (sLe^x) epitope on the acute phase protein α 1-acid glycoprotein (De Graaf et al., 1993) which is expressed in abomasal mucosa, and also known to upregulate with *T. circumcincta* infection (personal communication, Gillian Goldsworthy). Indeed, the results of carbohydrate histochemistry presented in this chapter might suggest that changes in SIAT4C expression were not related to mucus acidification. Further work

is required to determine if changes in SIAT4C expression are related to changes in acidification of the mucins in this model.

Of particular interest is the mechanism of regulation of SIAT4C expression. Previous results, had shown a downregulation of SIAT4C in response to IL-4 in sheep tracheal explants (chapter 5) and upregulation in response to TNFα in human bronchial explants stimulated with the proinflammatory cytokine, TNFα (Delmotte et al., 2002). More recently IL-1β has been shown to upregulate SIAT4C gene transcription in the HuH-7 hepatic carcinoma cell line (Higai et al., 2006). In contrast to ITLN, it is possible that any upregulation of SIAT4C in this model of *T. circumcincta* infection is secondary to a pro-inflammatory response rather than a Th2 response. Further work is required to support this hypothesis.

6.5 Conclusion

T. circumcinta challenge infection results in a Th2 response demonstrated by increased transcript expression of markers for mast cells (sMCP-1) eosinophils (OvGal-14) and the Th2 cytokine, IL-4. The upregulation of sITLN transcripts and protein in abomasal mucosa in response to *T. circumcincta* challenge infection, and upregulation of transcript at an earlier time point in cpi yearlings and lambs compared to cnv groups, is consistent with a protective role for sITLNs in the immune response to *T. circumcincta*. The upregulation of sSIAT4C in cpi sheep and lambs, but not in cnv animals, when compared to unchallenged groups, may support a role for SIAT4C in the protective immune response to *T. circumcincta*. For some transcripts, there was a difference in transcript expression between yearling sheep and lambs, in response to challenge infection with *T. circumcincta*, which may suggest that age does affect response to challenge infection.

7 Transcript expression in T. circumcincta susceptible and resistant lambs

7.1 Summary

The transcript expression of sheep ITLN genes (sITLN1, sITLN2, sITLN3) and genes considered markers of a Th2 response (sMCP-1, IL-4, OvGal-14) was examined in the abomasal mucosa of control lambs (n = 10) and lambs trickle infected with *T. circumcincta* for 16 weeks (n = 47). Lambs had been bred based on phenotypic selection for susceptibility and resistance to *T. circumcincta*. Lambs were ranked on post mortem *T. circumcincta* worm count and three groups established: control (C), high worm count (H) and low worm count (L); for each group n = 10. Relative transcript expression was significantly increased in groups H and L compared to group C for all genes except for IL-4. There was no significant difference between groups H and L for any transcript. Lambs had a concurrent infection with coccidia which may have affected transcript expression.

7.2 Introduction

Previous research has shown that there is a difference in resistance to gastro-intestinal parasites between and within sheep breeds (Stear and Murray, 1994; Douch et al., 1996; Bouix et al., 1998; Amarante et al., 2004). Considerable research has focused on *T. circumcincta* which is the dominant sheep nematode in temperate climates (Stear et al., 1998). There is evidence that experimental trickle infection with *T. circumcincta* confers a degree of immunity (Smith et al., 1983a). The present experimental protocol was designed to mimic a natural infection with *T. circumcincta*. Scottish blackface lambs were trickle infected with *T. circumcincta* L3 for a 16 week period before being killed and abomasal worm burden assessed. Lambs were bred for a spectrum of susceptibility and resistance to *T. circumcincta* based on previous phenotyping of parental stock (Davies et al., 2006). The

experiment was undertaken at the University of Edinburgh and abomasal mucosal tissue was kindly made available courtesy of Professor Josephine Pemberton Ashworth Building, Kings Buildings and Professor John Hopkins, Preclinical Sciences, Summerhall Square. Permission was given to examine transcript expression of the mucus associated molecules sITLN1, sITLN2 and sITLN3 which may play a role in the immune response to *T. circumcincta*.and also transcript expression of known markers of a Th2 response; sMCP-1, OvGal-14 and IL-4. Full details of all materials and methods are given in chapter 2. A Kruskal-Wallis test was used to assess significant difference between groups and a Mann-Whitney U was used as a post hoc test for all pairwise comparisons. $P < 0.05$ was considered significant. Spearman's rank correlation was used for all correlations.

7.3 Results

7.3.1 Transcript expression in abomasal mucosa

7.3.1.1 Introduction

The experimental protocol and source of animals (2.1.6, 2.2.4) has been described previously. In brief, there were 57 lambs in this experimental group, 47 of which were exposed to trickle infection with *T. circumcincta* over approximately 16 weeks and 10 lambs remained worm free and were used as controls. Every two weeks during trickle infection faeces were collected for nematode faecal egg count and coccidial oocyst count. When infection with coccidia was found, all animals were treated with a commercial coccidiostat. Animals were killed after the 16 week trickle infection, abomasal worms counted and abomasal mucosa collected for determination of transcript expression (2.2.6 - 2.2.8,). The lambs were ranked according to abomasal worm counts and grouped as follows: group C (controls); group L (trickled infected low worm count); group H (trickle infected high worm counts). The expression of sITLN1, sITLN2 and sITLN3 transcripts in the abomasal mucosa was determined in all lambs (2.3.1.2, 2.3.4 - 2.3.8) and then transcript expression in groups C, L and H was compared. Furthermore, in order to characterise the model, expression of transcripts typical of a Th2 response (sMCP-1, OvGal-14

and sIL-4), were examined concurrently. Total worm counts were correlated with transcript expression. Furthermore, transcript expression was also correlated with the final faecal coccidial oocyst count, which was taken 1-2 weeks before the lambs were killed.

7.3.1.2 Abomasal Teladosagia circumcincta worm counts and final faecal coccidial oocyst counts

There were no *T. circumcincta* worms present in the abomasum of the 10 control lambs (group C). There were *T. circumcincta* worms present in 37/47 of the trickle infected lambs (group TI). The 10 trickle infected lambs with no worms present and the 10 trickle infected lambs with the highest worm counts were termed groups L and H respectively (Figure 7.1 A, B). Coccidial oocysts were present in control (8/10) and trickle infected lamb faeces (38/47). There was no significant difference in coccidial occyst counts between groups C and TI ($p = 0.8666$). A Kruskal-Wallis test showed a significant difference in coccidial oocyst burden between groups C, L and H ($p = 0.0038$). Pairwise comparison using Mann-Whitney U showed a significant difference between groups C and L ($p = 0.0433$) and groups L and H ($p = 0.0003$) but not between groups C and H ($p = 0.2475$) (Figure 7.1 C, D) Coccidial oocyst burden and worm burden showed a significant weak negative correlation (Figure 7.1 E).

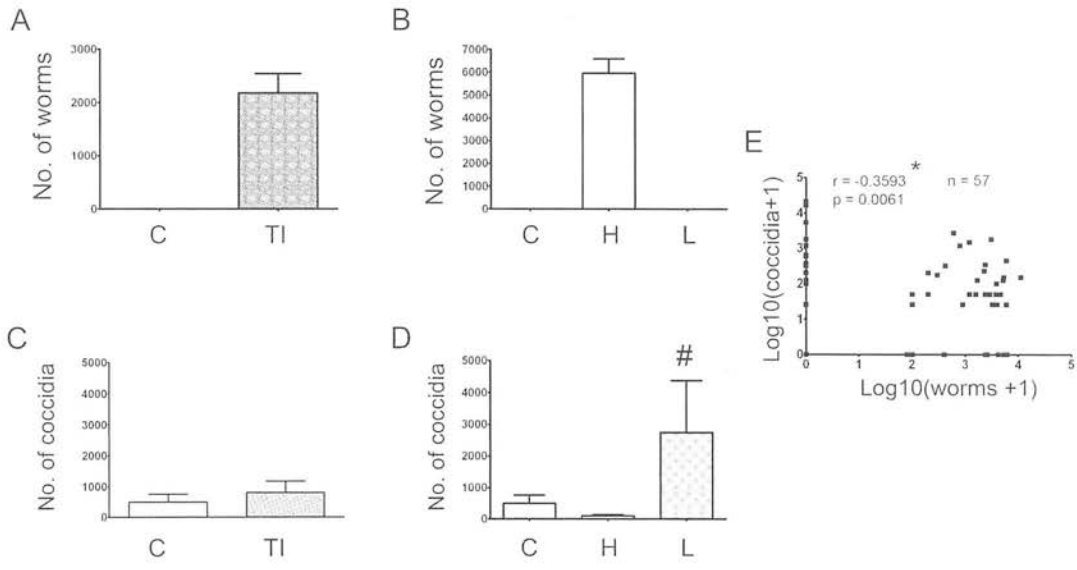


Figure 7.1: Abomasal *Teladosagia circumcincta* adult worm counts and faecal coccidial oocyst counts

Graphs A & B show number of adult *T.circumcincta* worms recovered from the abomasum of the following lamb groups; 10 control lambs (C), 47 trickle infected lambs (TI), 10 lambs with highest worm counts (H) and 10 lambs with lowest worm counts (L).

Graphs C & D show number of coccidian occysts recovered from faeces of lamb groups C, TI, H and L. A Kruskal-Wallis test showed significant difference between groups and a Mann-Whitney U test was used for pair wise comparisons. # represents significant difference compared to C and H. Significance was set at $p < 0.05$.

Graph E shows correlation between worm burden and faecal coccidian burden. Asterix represents significant correlation. Bars represent mean and S.E.M

7.3.1.3 Expression of sITLN transcripts in abomasum of all lambs using RT-PCR

sITLN1, sITLN2 or sITLN3 transcripts were not expressed in the abomasal mucosa of group C lambs. There was variable expression of sITLN1 (30/47), sITLN2 (35/47) and sITLN3 (36/47) transcripts in the 47 trickle infected lambs. Some lambs expressed all 3 sITLNs (18/47) whilst others expressed one (15/47) or two sITLNs (14/47). In general, expression of sITLN1 transcript expression was low or absent

whilst expression of sITLN2 and sITLN3 was stronger. Transcript expression of the house keeping gene sheep ATPase was relatively constant. Within the trickle infected group of lambs, individual lambs showed marked variation in transcript expression for all three sITLNs (Figure 7.2 A).

7.3.1.4 Comparison of expression of sheep ITLN transcripts in abomasal mucosa of lamb groups (C, L and H) using semi-quantitative RT-PCR

Relative transcript expression of sITLN1, 2 and 3 was compared in groups C, L and H and a significant difference was found between groups for all three sITLNs. Pairwise comparison, showed a significant difference in transcript expression between groups C and L, and groups C and H for sITLN1, 2 and 3, There was no significant difference in transcript expression between groups L and H (Figure 7.2B, Table 7.1). Interestingly for sITLN2, transcript expression was more consistently observed in group L (9/10) compared to group H (5/10), however a Chi squared test showed no significant difference ($\chi^2 = 1.63$, $p = 0.45$) and lambs in group L were more likely to express all 3 ITLNs (6/10) when compared to lambs in group H (3/10) again the Chi squared test show no significant difference ($\chi^2 = 1.53$, $p = 0.45$) (Figure 7.2A).

7.3.1.5 OvGal-14, sIL-4 and sMCP-1 transcript expression in abomasal mucosa of all lambs using RT-PCR

OvGal-14 transcript was expressed in the abomasal mucosa of only 1/10 lambs in group C, whilst expression of sIL-4 transcript was seen in 8/10 lambs and sMCP-1 transcript expression was also seen in 8/10 lambs in this group. OvGal-14 transcript was expressed in 39/47 trickle infected lambs, whilst sIL-4 and sMCP-1 transcript was expressed in the abomasal mucosa of all 47 trickle infected lambs (Figure 7.2A).

7.3.1.6 Comparison of OvGal-14, sIL-4 and sMCP-1 transcript expression in abomasal mucosa of lamb groups (C, L and H) using semi-quantitative RT-PCR

Relative transcript expression of OvGal-14, sIL-4 and sMCP-1 was compared in groups C, L and H using a Kruskal-Wallis test and a significant difference was found between groups for OvGal-14 and sMCP-1 but not for sIL-4. Pairwise comparison showed a significant difference in OvGal-14 and sMCP-1 transcript expression between groups C and L and groups C and H, whilst no significant difference was found between groups L and H (Figure 7.2B, Table 7.1). Interestingly, OvGal-14 transcript was expressed in 10/10 group L lambs whilst expression was present in only 6/10 lambs in group H, however a Chi squared test showed no significant difference between groups ($\chi^2 = 1.42, p = 0.45$) (Figure 7.2A).

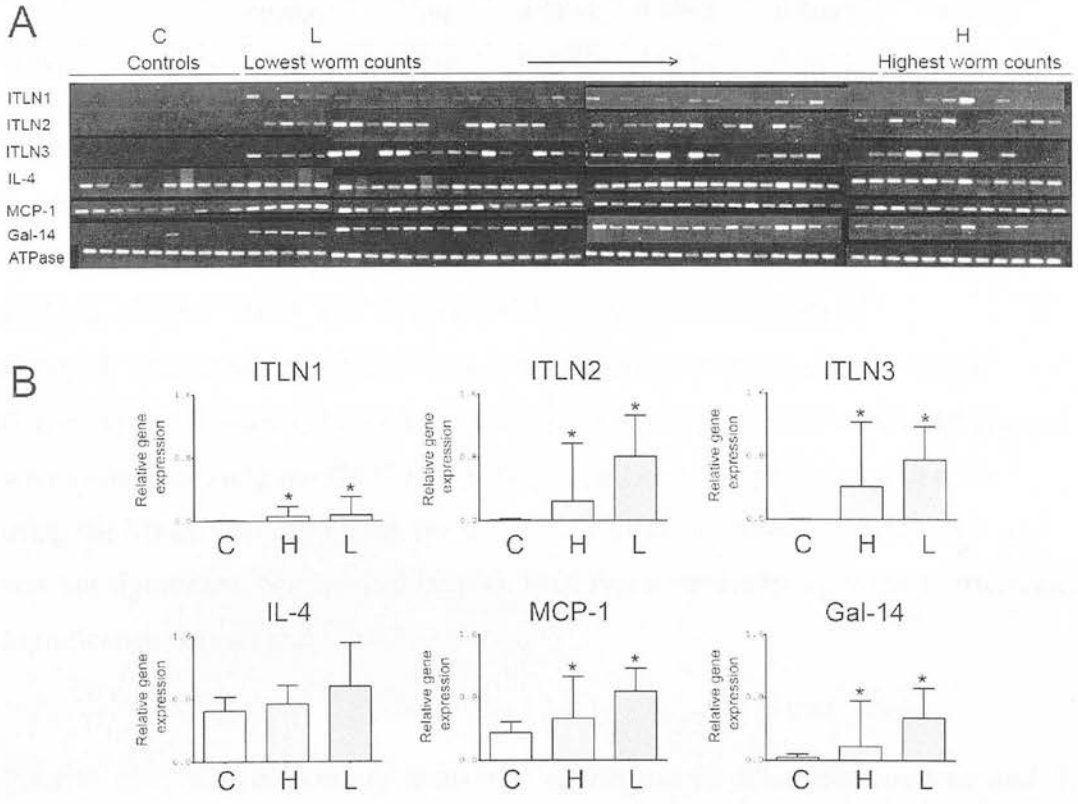


Figure 7.2: Transcript expression in abomasal mucosa of lambs

A. Ethidium bromide gel showing transcript expression of sITLN1, sITLN2, sITLN3, sIL-4, sMCP-1, OvGal-14 (Gal-14) and the house keeping gene sheep

ATPase in abomasal mucosa of control lambs (n = 10) and lambs (n = 47) after trickle infection with *T. circumcincta*.

B. Relative transcript expression of sITLN1, sITLN2, sITLN3, sIL-4, sMCP-1, OvGal-14 in abomasal mucosa of three groups of lambs, for each group n=10. The groups were as follows: C (controls), H (highest worm counts), L (lowest worm counts). Groups H and L were trickle infected with *T. circumcincta* for 16 weeks. Bars represent mean and S.E.M. A Kruskal-Wallis test showed significant difference between groups and a Mann-Whitney U test was used for pair wise comparisons. * represents significant difference compared to controls. Significance was set at $p < 0.05$.

	Ovgal 14	IL-4	sMCP-1	ITLN1	ITLN2	ITLN3
Kruskal -Wallis	**0.0004**	0.0819	**0.0046**	**0.0058**	**0.0014**	**0.0004**
C v H	**0.0089**	n/a	**0.0355**	**0.0147**	**0.0115**	**0.0021**
C v L	**<0.0001**	n/a	**0.0011**	**0.0015**	**0.0003**	**<0.0001**
H v L	0.0630	n/a	0.1903	0.6842	0.1431	0.3930

Table 7.1: Statistical results for comparison of relative transcript expression in abomasal mucosa of groups of lambs.

Statistical results for comparison of relative transcript expression of sITLN1, sITLN2, sITLN3, sIL-4, sMCP-1, OvGal-14, in abomasal mucosa of Groups C (controls), H (highest worm counts), L (lowest worm counts) lambs. Groups H and L were trickle infected with *T. circumcincta* for 16 weeks. All groups were compared using the Kruskal-Wallis test; pairwise comparisons were made using the Mann-Whitney U test. No post hoc test was undertaken if Kruskal-Wallis was not significant. Not applicable (n/a). Bold types represents significant difference. Significance was set at $p < 0.05$.

7.3.1.7 Correlations of transcript expression in abomasal mucosa and T. circumcincta worm counts

Transcript expression in the abomasal mucosa for sITLN1, sITLN2, sITLN3, IL-4, sMCP-1 and Ovgal-14 was correlated with *T. circumcincta* worm counts. Transcript was expressed relative to the house keeping gene sheep ATPase. Spearman's rank

correlation was used, as data were not normally distributed. There was a trend to a negative correlation between some genes and worm counts; however, it was only for OvGal-14 that a weak significant negative correlation was shown (Figure 7.3). Worm data is shown on graphs as log10 (worms +1) for graphical purposes. Sheep ATPase was the house keeping gene.

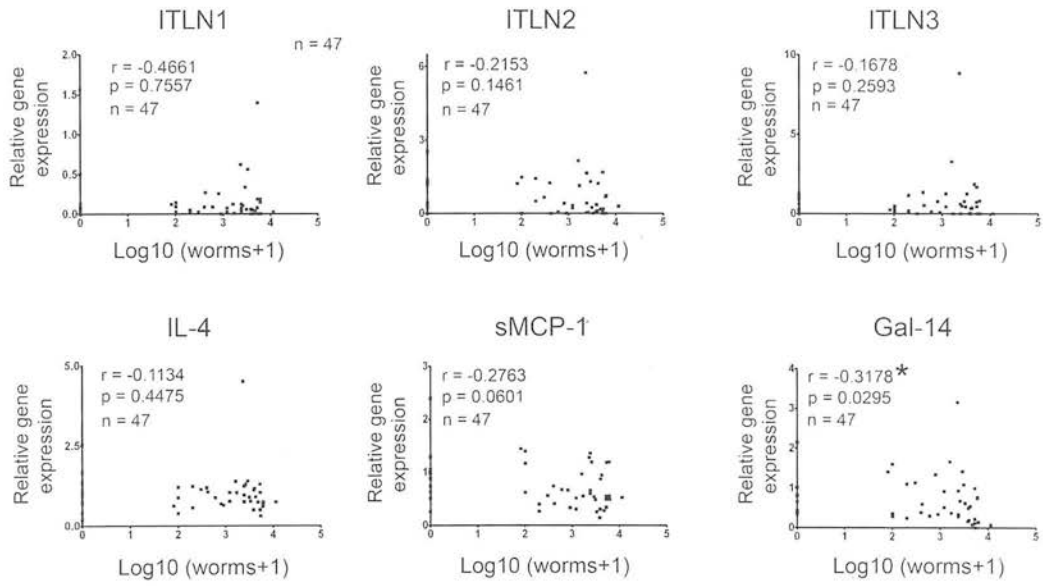


Figure 7.3: Correlation of transcript expression in abomasal mucosa of trickle infected lambs and *T. circumcincta* worm counts

Correlation of transcript expression, of sITLN1, sITLN2, sITLN3, IL-4, sMCP-1, OvGal-14 and log 10 (worm counts + 1). Transcript is expressed relative to the house keeping gene sheep ATPase. * represent significant negative correlation. A Spearman's rank correlation was used.

7.3.1.8 Correlations of transcript expression in abomasal mucosa of trickle infected lambs and faecal coccidial oocyst counts

Because of the possibility that coccidial oocyst counts might influence transcript expression in the abomasal mucosa, transcript expression for sITLN1, sITLN2, sITLN3, sIL-4, sMCP-1 and Ovgal-14 in trickle infected lambs was correlated with faecal coccidial oocyst counts. Spearman's rank correlation was used, as data were

not normally distributed. There was a significant weak positive correlation between Ovgal-14 transcript expression and coccidial oocyst counts (Figure 7.4). Coccidial oocyst counts are shown on graphs as log10 (coccidial oocyst counts +1) for graphical purposes. Transcript is expressed relative to the house keeping gene sheep ATPase.

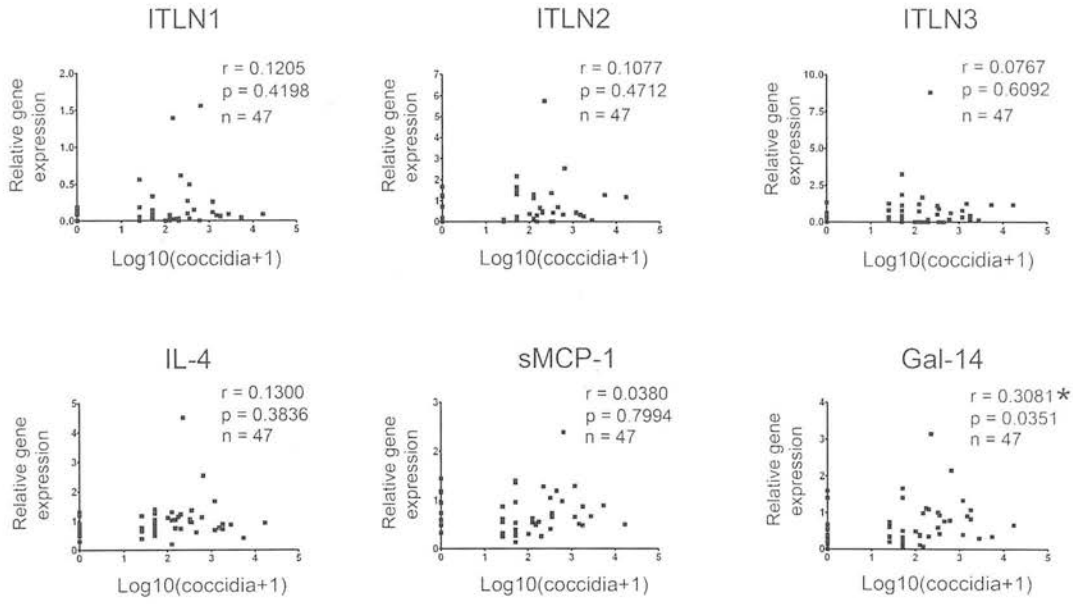


Figure 7.4: Correlation of transcript expression in abomasal mucosa of trickle infected lambs and coccidial oocyst counts

Correlation of transcript expression of sITLN1, sITLN2, sITLN3, IL-4, sMCP-1, OvGal-14 and log 10 (coccidial oocysts + 1). Transcript is expressed relative to the house keeping gene sheep ATPase. * represents significant positive correlation using Spearman's rank test.

7.4 Discussion

7.4.1 Experimental model

Control of nematode infections in the livestock industry, has until recently relied on a small number of highly effective drugs. As drug resistance increases (Wrigley et al., 2006; Hughes et al., 2007; Traversa et al., 2007; Wilson and Sargison, 2007),

alternative control strategies are being explored including breeding for resistance (Bouix et al., 1998; Stear et al., 2007a; Stear et al., 2007b) and vaccination (Hein and Harrison, 2005; Knox and Redmond, 2006; Smith and Zarlenga, 2006). In the present experiment, the selective breeding of sheep that had been phenotyped for low and high faecal egg counts was successful in producing a group of lambs that showed a wide spectrum of susceptibility and resistance to *T. circumcincta*. The selection of female lambs only, removed the natural variance due to sex (Craig et al., 2006) and the use of a single breed reduced the natural variation between sheep breeds (Miller and Horohov, 2006) . There is considerable variation between sheep of the same breed in response to *T. circumcincta* infection (Crawford et al., 2006)Crawford, 2006) and it could be argued that the range of response to trickle infection found in these lambs is no greater than would occur in any group of lambs without prior phenotyping of the breeding stock.

7.4.2 Coccidial infection

Coccidial infections are common in sheep, a variety of species have been identified and infections are typically more intense in young animals (Amarante and Barbosa, 1992; Alzieu et al., 1999; Reeg et al., 2005; Craig et al., 2008). Infection may cause clinical disease or may be sub-clinical and result in poor productivity (Gregory and Catchpole, 1989; Berriatua et al., 1994; Alzieu et al., 1999). Infections typically involve the small intestine, however there are reports of infection of the abomasum resulting in clinical disease (Maratea and Miller, 2007). The present experiment was designed to examine the immunological response to *T. circumcincta,* a known inducer of a Th2 response, and concurrent infection of the experimental lamb flock with coccidia was unfortunate. Protozoan parasites typically induce a Th1 biased response in ruminants (Hermosilla et al., 1999; Maley et al., 2006) however there is evidence from other species that mixed Th1/Th2 responses may occur (Schito et al., 1998; Nishikawa et al., 2003; Hong et al., 2006).

Increased globular leucocytes (intra-epithelial mast cells) has been shown in coccidial infections of lambs (Gregory and Nolan, 1981), and increased mast cells

and eosinophils have been shown in selected coccidial infections in rodents (Shi et al., 2000). It cannot be ruled out that some of the changes in the transcripts noted in the present experiment, in particular in the control animals, were due to presence of coccidia within the abomasum or alternatively were secondary to a generalised mucosal response to infection with coccidia.

Of particular interest was the significant weak negative correlation between faecal oocyte count and *T. circumcincta* worm burden. In natural conditions worm and protozoal infections are mixed and there is considerable interest in the immunological response to co-existing infections (Graham, 2002; Craig et al., 2008). Typically a positive correlation has been shown between nematode worm counts and faecal oocyst counts (Catchpole and Harris, 1989; Agyei, 2003) and further work is required to examine the negative correlation found in these lambs.

7.4.3 Expression of sITLN transcripts in abomasal mucosa of lambs

The absence of expression of sITLN transcripts in control lambs and presence in *T. circumcincta* trickle infected lambs, would suggest that ITLN upregulation is nematode specific. However, there was no significant correlation between any sITLN transcript and abomasal worm counts and upregulation of sITLNs did not differ significantly in lambs with high and low *T. circumcincta* worm counts. This finding in itself would support the suggestion that upregulation of ITLNs is not protective (Voehringer et al., 2007). Putative functions of ITLNs include binding of bacteria (Tsuji et al., 2001) and alteration of mucus character (Nishihara et al., 1986). ITLNs may be acting as an antiseptic coating in the worm damaged abomasal mucosa. Alternatively, it is possible, that by altering the character of mucus, ITLNs may lead to entrapment of worms and thus permit effector cells or molecules to act on parasites. It is of interest that sITLN transcripts, which *in vitro* have been shown to be highly sensitive to IL-4, were not expressed in the abomasal mucosa of control sheep despite the expression of IL-4 transcript. Using transgenic and knock out mice ITLN expression has been shown to be STAT6 and IL-13 dependent in rodents

(Kuperman et al., 2005; Voehringer et al., 2007). IL-13 transcript expression was not assessed in the present model, however it could be postulated that *in vivo* regulation of ITLNs in sheep might be predominantly IL-13 mediated.

7.4.4 Expression of IL-4 transcript in abomasal mucosa of lambs

IL-4 transcript was expressed in the abomasal mucosa of the control lambs in the present experiment. It is unknown whether this is a normal finding in lambs or if it was related to concurrent infection with coccidiosis which has been shown in some species to induce a mixed Th1/Th2 reponse (Nishikawa et al., 2003; Hong et al., 2006). There was no correlation between IL-4 transcript expression and coccidial oocyst counts in the trickle infected lambs, which would argue against any relationship between IL-4 expression and coccidial oocyst count. IL-4 transcript expression in the abomasal mucosa did not differ between groups C, H and L, a finding which is in agreement with previous work with *Haemonchus contortus* challenge infection of susceptible (INRA 410) and resistant (Barbados black belly) breeds of sheep (Terefe et al., 2007). However, the tissue examined, appears to affect results, as in the same experiment with *H. contortus*, a significant difference was found between groups for IL-4 transcript expression in abomasal lymph nodes (Terefe et al., 2007).

7.4.5 Expression of sMCP-1 transcript in abomasal mucosa of lambs

Mast cells are considered important effector cells in expulsion of some nematode parasites in particular *T. spiralis* in mice (Vliagoftis, 2005; Miller, 1996) and are markers of a Th2 response. Activation leads to degranulation and release of histamine, heparin and proteases, however, mast cells can also secrete cytokines (IL-4, TNFα), leukotrines and chemokines (de Veer et al., 2007). In the present experiment there was significantly higher expression of sMCP-1 transcript in trickle infected lambs compared to controls, which most likely reflects an increased number of mucosal mast cells/ globule leukoctyes. Interestingly, no significant difference in sMCP-1 transcript expression was shown between groups H and L, and no

correlation was found between number of worms and sMCP-1 transcript expression. In mice mMCP-1 expression is required for nematode expulsion and deficiency results in impaired expulsion, it is unknown if the same applies to sheep (Knight et al., 2000). All trickle infected lambs in the present experiment expressed sMCP-1. The expression of sMCP-1 transcript in several of the control lambs is of interest and it is unknown if this expression could have been related to coccidial infection. Previous studies have shown that abomasal nematode infection can lead to sMCP-1 upregulation in small intestine mucosa (Huntley et al., 1995) and thus, it is possible, that small intestinal coccidial infection might lead to increased sMCP-1 expression in the abomasal mucosa. Mast cells can secrete IL-4 and it is possible that mast cells represented a source of IL-4 in the present experiment.

7.4.6 Expression of Ovgal-14 transcript in abomasal mucosa of lambs

Eosinophils are increased locally and systemically in parasitic infections, however there is considerable debate as to whether they play a protective role (Else and Finkelman, 1998; Rothenberg and Hogan, 2006; de Veer et al., 2007). Ovgal-14 has been shown to be a marker of sheep eosinophils and it is unknown if it has a specific role to play in protection against parasitic infections (Dunphy et al., 2002; Huntley et al., 2005). In the present experiment OvGal-14 transcript expression was significantly increased in trickle infected lambs compared to controls and furthermore a significant weak negative correlation was shown between worm number and transcript expression. Transcript expression was higher in group L compared to group H lambs however this failed to reach significance. These findings are supportive of a relationship between resistance to *T. circumcinta* and eosinophils. Interestingly, eosinophils have been reported in coccidial infections in lambs and a significant weak positive correlation was shown between coccidial oocyst number and OvGal-14 transcript expression.

7.5 Conclusion

Trickled infection with *T. circumcincta* provided enough stimulus to result in increased transcript expression of sMCP-1, OvGal-14, sITLN1, sITLN2 and sITLN3 compared to control lambs. There was no significant difference in transcript expression between lambs with high and low worm counts suggestive that increased expression was not protective against nematodes. However, there was a significant weak negative correlation between worm counts and OvGal-14, which might support a protective role for eosinophils. The concurrent infection with coccidia may have had an affect on transcript expression.

8 General Discussion

8.1 Mucosal immune response

Th2 cytokine induced inflammatory responses play a role in defence against helminth infection (Urban et al., 1998; Onah and Nawa, 2000; Grencis, 2001) but are also responsible for the pathogenesis of several diseases, including asthma, allergy and inflammatory bowel disease (Neurath et al., 2002; Walter and Holtzman, 2005; Neurath and Finotto, 2006). Understanding the molecules and pathways of Th2 inflammation is essential for identification of new treatment strategies. Goblet cell hyperplasia is a feature of Th2 inflammation and recent gene profiling studies have identified ITLN, RELMβ and SIAT4C as mucus assocciated molecules highly upregulated in Th2 inflammation (Knight et al., 2004; Pemberton et al., 2004a; Datta et al., 2005; Artis, 2006; Yamauchi et al., 2006; Kawai et al., 2007). The experimental work presented in this thesis has explored the hypothesis that these three mucus associated molecules are co-regulated in a Th2 environment. *In vitro* experiments were undertaken in human and sheep cell culture systems whilst *in vivo* experiments were undertaken in sheep. Sheep were chosen because of the economic importance of parasitic infections in sheep and because of the use of sheep as an experimental model for respiratory diseases including asthma in humans.

8.2 RELMβ

The recently described RELM/FIZZ family, of which there are four described in rodents (RELMα RELMβ, Resistin, RELMγ) and two in humans (RELMβ, Resistin) are of considerable interest. Expression of RELMα and RELMβ has been shown in a Th2 environment in the gastrointestinal and respiratory tract of rodents (Holcomb et al., 2000; Artis, 2006; Mishra et al., 2007). Interestingly RELMα is expressed in a wide range of cells including epithelial cells, alternatively activated macrophages, B lymphocytes and dendritic cells (Loke et al., 2002; Nair et al., 2006) whilst RELMβ

expression has been related to mucus producing cells (Artis et al., 2004). RELMβ expression has been shown in the colon of humans (He et al., 2003) and a human colonic mucoid adenocarcinoma cell line was used for the majority of the experimental cell culture work presented in this thesis. Experimental results presented here confirmed the upregulation of RELMβ following incubation with either of the Th2 cytokines IL-4 or IL-13, as had been shown previously (Artis et al., 2004). Furthermore, results demonstrated co-regulation with the mucus associated molecule ITLN in this cell culture line. Interestingly expression of both RELMβ and ITLN has been shown to be STAT6 dependent (Stutz et al., 2003; Voehringer et al., 2007). Attempts to identify RELMβ transcript in sheep tissue were unsuccessful, however resistin transcript was found to be expressed (personal communication, Pam Knight), indicating that some of the RELM family members are present in sheep. Resistin which is expressed in adipocytes in mice and macrophages in people (Curat et al., 2006) has been studied extensively for its putative links with obesity and type II diabetes (Steppan et al., 2001a; Degawa-Yamauchi et al., 2003; Banerjee et al., 2004). Whilst a role has been shown for resistin as a biomarker of inflammatory disease in man (Reilly et al., 2005; Senolt et al., 2007; Sunden-Cullberg et al., 2007) to date no links have been shown with Th2 inflammatory disease. As the focus of this thesis was mucus associated molecules upregulated in Th2 inflammation, no further work was undertaken with the RELM family of molecules in sheep.

8.3 SIAT4C

Goblet cells secrete high molecular weight, heavily glycosylated mucin proteins (Rogers, 2003). Alteration in the glycosylation of mucins has been demonstrated in a range of respiratory and gastro-intestinal diseases in man and rodents including bacterial and parasitic infections (Koninkx et al., 1988; Ishikawa et al., 1993; Davril et al., 1999; Corfield et al., 2001; Holmen et al., 2002; Theodoropoulos et al., 2005; Yamauchi et al., 2006). A role for mucin glycosylation in resistance against parasitic infections in rodents has been proposed (Ishikawa et al., 1993; Ishikawa et al., 1994; Maruyama et al., 2002). Transient alteration in sialylation has been reported in parasitic infections in rodents which normalised after expulsion of parasites

(Karlsson et al., 2000) and upregulation of SIAT4C transcript has been shown in Th2 type parasitic infections in rodents (Knight et al., 2004; Yamauchi et al., 2006; Kawai et al., 2007). In the experimental cell culture work presented in this thesis using a human colonic adenocarcinoma cell line (LS 174T) and a human respiratory mucoepidermoid carcinoma cell line (NCI-H292), expression of SIAT4C transcript was not altered following culture with the Th2 cytokines IL-4, IL-9 or IL-13.. As upregulation of SIAT4C had been shown in Th2 type parasitic infections in rodents these results were unexpected.

Expression of SIAT4C transcript had not been shown previously in sheep tissue and thus before undertaking any experimental work is sheep, transcript expression was first confirmed in sheep abomasal tissue using primers designed from the bovine sequence. Subsequently, following release of EST data from New Zealand in 2006, the full sheep sequence was deduced and transcript expression was shown in a wide range of sheep tissues as previously had been found in other mammalian species (Varki et al., 1999). No attempt was made to determine if alternatively sliced variants of SIAT4C were present in sheep as had been reported in man, however, there was some evidence from EST sequences deposited in Genbank to suggest that variants may be present in sheep (Grahn and Larson, 2001).

In the sheep tracheal explant model presented in this thesis, SIAT4C transcript expression was significantly decreased by incubation with recombinant sheep IL-4. In the *T. circumcincta* challenge model, upregulation of SIAT4C transcript was shown in abomasal mucosa of sheep and lambs following challenge infection, as had been reported in nematode infections in rodents (Knight et al., 2004; Yamauchi et al., 2006). *In vitro* experiments have shown upregulation of SIAT4C transcript expression in response to incubation with TNFα or IL-1β (Delmotte et al., 2001; Delmotte et al., 2002; Higai et al., 2006) however upregulation by TNFα was not observed in the *in vitro* cell culture work presented in this thesis and the effect of incubation with IL-1β was not assessed. Interestingly a higher concentration of TNFα was used in previously published work because of a known concentration effect of TNFα on mucin and fucosyltransferase expression which may also be true for

SIAT4C (Levine et al., 1995; Delmotte et al., 2002) and this may explain the differing results.

Interestingly whilst the cytokine profile of gastric lymph node of the yearling sheep following challenge infection with *T. circumcincta* used in these experiments had a strong Th2 bias, concurrent upregulation of TNFα and IL-1β was also present (Craig et al., 2007). TNFα has been implicated in resistance to nematode infections in rodents (Artis et al., 1999; Hayes et al., 2007). It is possible that any change in expression of SIAT4C in parasitic infections is more associated with a pro-inflammatory response than a Th2 type response. A particularly interesting finding in the MRI *T. circumcincta* challenge model was the significant upregulation of SIAT4C in cpi yearlings and lambs but not in cnv yearings and lambs, is consistent with a protective role for SIAT4C expression. Unfortunately, permission was not obtained to examine expression of SIAT4C in the University of Edinburgh lamb model of *T. circumcincta* trickle infection, which might have clarified the role of SIAT4C in resistance to nematode infection.

Changes in SIAT4C transcript expression did not appear to correlate with acidification of mucins as assessed by AB/PAS carbohydrate histochemistry either in the sheep tracheal explant model or in the *T. circumcincta* challenge infection model in the present experiments. As no antibody was available to immunolocalise SIAT4C expression, it cannot be assumed that changes in transcript expression were related to goblet cell mucin sialylation. Indeed, in man SIAT4C has been implicated in selectin ligand formation and lymphocyte trafficking (Ellies et al., 2002b; Sperandio et al., 2006) and increased expression of SIAT4C has been shown in activated T cells. (Blander et al., 1999). Lectin histochemistry is another methodology for examination of changes in glycosylation. The lectin, *Maackia amurensis* agglutinin (MAA), is specific for sialic acid in a 2-3 linkage, however as several sialyltransferases exist that link sialic acid in a 2-3 linkage, MAA was not considered specific enough to use in the present experiments. In situ hybridisation might have helped to localise upregulated transcript but was not attempted due to time limitations.

8.4 ITLNs

ITLNs are recently discovered mucus associated molecules which have been shown to upregulate in a Th2 environment in the respiratory and gastro-intestinal tract of rodents and respiratory tract of people (Pemberton et al., 2004a; Pemberton et al., 2004b; Datta et al., 2005; Kuperman et al., 2005; Artis, 2006; Voehringer et al., 2007). The results of the cell culture experiments presented in this thesis, which used a human mucoid colonic adenocarcinoma cell line, demonstrated upregulation of ITLN following culture with recombinant IL-4 or IL-13 as had been shown previously (Julie Bethune, MSc thesis, University of Edinburgh 2005) and furthermore demonstrated that upregulation was dose and time dependent. At the start of this experimental work it was known that ITLN was present in sheep (Alan Pemberton, personal communication), however it was unknown if more than one ITLN was expressed as had been shown in mice and man (Lee et al., 2001; Pemberton et al., 2004a).

Three distinct sheep ITLNs were sequenced and cloned and submitted to GenBank. Differential transcript expression in tissues has been shown for mouse and human ITLNs (Lee et al., 2001; Pemberton et al., 2004a; Suzuki and Lonnerdal, 2004) and the results of the present experiments show that the findings in sheep are similar with expression of sITLN2 restricted to abomasum in normal sheep, whilst sITLN1 and sITLN3 showed a much wider tissue distribution. Some sheep did not express sITLN2 and sITLN3 and it is unknown if this was due to deletions within the ITLN locus as has been found in certain strains of mice (Pemberton et al., 2004a). Comparative Southern blots of genomic DNA might have helped to clarify this finding, however they were not undertaken due to time restriction.

Regulation of sITLNs by Th2 cytokines was shown in the sheep tracheal explant model and the sheep respiratory epithelial cell culture model in this thesis, as had been demonstrated in the human LS174T cell culture line. Unfortunately, due to variation in expression of sITLNs between sheep and the low numbers of sheep,

statistically significant upregulation was not demonstrated in the tracheal explant model. No statistical analysis was done on the sheep respiratory epithelial cell culture model as only a limited number of samples was available; however, a clear trend to upregulation of sITLNs was demonstrated in both model systems.

Natural infection with *D. filaria* and challenge infection with *T. circumcincta* resulted in unequivocal significant upregulation of sITLN transcripts compared to controls as had been shown in nematode infections of mice (Pemberton et al., 2004a; Datta et al., 2005; Voehringer et al., 2007). For *D. filaria* infection significant upregulation was present for sITLN2 and sITLN3 in the lung tissue compared to uninfected controls whilst for *T. circumcincta* challenge infection significant upregulation was seen for all three sITLNs in cpi compared to upi sheep with significant upregulation seen at an earlier time point in cpi compared to cnv sheep. The earlier upregulation of potential effector molecules in cpi (immune) animals compared to cnv animals has been described previously, however it does not necessarily imply a protective role.

Sheep ITLNs were shown to be significantly upregulated following trickle infection of lambs bred with variable resistance to *T. circumcincta* compared to control lambs; however, no significant difference was shown between lambs considered resistant and susceptible to *T. circumcincta* for any of the sITLNs, which would support the suggestion that ITLN expression is not in itself protective for nematode infection. As only transcript expression was determined further work examining protein expression is required using either immunohistochemistry or Western blot analysis of abomasal tissue to support this suggestion. The affinity purified chicken antibody to ITLN peptide 1 is not selective and identifies all sheep ITLNs. However as sITLN2 transcript appeared to be the most upregulated ITLN in resistant sheep, ideally production of specific antibodies for individual sheep ITLNs would be ideal. Based on the sequence data from this experimental work, a sITLN2 recombinant protein has been developed at the Moredun Research Institute and a specific antibody is presently being developed.

8.5 Markers of a Th2 response

The focus of this thesis has been novel mucus associated molecules upregulated in Th2 type responses in the mucosal membranes. At the start of experimental work whilst *T. circumcincta* was known to induce mucosal mastocytosis, peripheral eosinophilia, mucosal eosinophil infiltration and increased IgE (Stevenson et al., 1994; Huntley et al., 1998; Scott et al., 2000); all markers of a Th2 response, the cytokine profile during infection had not been published. Thus, in order to characterise the *T. circumcincta* sheep models used, expression of transcripts representative of a Th2 response, for IL-4, sMCP-1 and OvGal-14 were examined, and these results merit some discussion.

IL-4 plays an important role in differentiation of Th2 cells and is a signature cytokine produced by these cells (Else and Finkelman, 1998). The significant upregulation of IL-4 transcript in abomasal mucosa of *T. circumcincta* challenged yearling sheep confirms a Th2 response to this nematode. Interestingly there was no significant upregulation of IL-4 transcript in lambs following challenge infection, which would support an age related difference in immune response. A comprehensive study is being undertaken of the cytokine response in the abomasal mucosa of the yearling sheep and lambs from the MRI challenge model, which will provide further information on the effect of age on immune response (personal communication, Nicky Craig). In the UoE lamb trickle infection model there was no significant difference in expression of IL-4 transcript between resistant and susceptible lambs. This result may have been affected by the concurrent infection with coccidiosis, however the finding is in agreement with published findings for *Trichostrongylus colubriformis* infections where, of the cyokines examined, only IL-13 and IL-5 expression was shown to differ between resistant and susceptible strains of sheep (Pernthaner et al., 2005).

OvGal-14 has been shown to be a specific marker of sheep eosinophils (Dunphy et al., 2002) and upregulation of transcript was present following challenge infection in

the MRI *T. circumcinta* model and following trickle infection in the UoE lamb model. In the MRI model, upregulation was seen in cpi and cnv sheep, however the kinetics differed, with earlier upregulation seen in the cpi sheep suggestive of control by the adaptive immune response. Of particular interest was the significant weak negative correlation between worm numbers and OvGal-14 transcript expression in the UoE trickle infected lambs, which might support a protective role for eosinophils. Despite several years of research on effector molecules in parasitic infections, it is still uncertain if eosinophils have a protective role to play in nematode infections (Onah and Nawa, 2000; Meeusen et al., 2005). Eosinophils can act as antigen presenting cells, communicate with mast cells and furthermore can also regulate T cell polarization (Rothenberg and Hogan, 2006). The relevance of many of these functions in nematode infection is unknown as is the function of the secreted lectin, OvGal-14 and further work is required to understand if this particular lectin has a specific role to play in the immune response in sheep nematode infections.

Increased mast cell numbers have been demonstrated in nematode infections of sheep (Gill et al., 2000; Huntley et al., 2004), supportive of a Th2 response and sMCP-1 has been shown to be a good marker of mast cells (Pemberton et al., 2000). In the *T. circumcincta* experiments presented in this thesis sMCP-1 was shown to be upregulated in the upi sheep, cpi sheep, and cnv sheep in the MRI *T. circumcincta* challenge model, supportive of a Th2 response. In lambs upregulation was only seen in cpi lambs; again evidence to support a different immune response in yearling sheep and lambs. In the UoE trickle infection model, no significant difference in sMCP-1 transcript expression was shown between susceptible and resistant lambs, however this result may have been affected by the concurrent coccidiosis. Mast cells have been shown to act as major effector cells of IgE mediated immediate hypersensitivity reactions leading to exclusion of parasites (Miller, 1996) However recent research has shown that mast cells have a role to play in both innate and adaptive immune responses. Mast cells express toll like receptors and can interact directly with bacteria (Vliagoftis and Befus, 2005). Mast cells can act as antigen presenting cells and can recruit inflammatory cells through release of pro-inflammatory mediators (Vliagoftis and Befus, 2005). Mast cells secrete proteases

and can affect mucosal permeability and they may play a role in tissue remodelling and fibrosis (Puxeddu et al., 2003; Lawrence et al., 2004). Further studies are required to fully understand the different roles that mast cells may play in nematode infections.

8.6 Conclusion

The experiments presented in this thesis have disproved the hypothesis that the mucus associated molecules ITLN, RELMβ and SIAT4C are co-regulated. *In vitro* experiments have demonstrated that ITLN and RELMβ are co-regulated however; SIAT4C is differentially regulated by Th2 cytokines. Upregulation of ITLN and SIAT4C has been shown in nematode infection of sheep which have a Th2 bias, as published in rodents. Interestingly, three sheep intelectins have been identified and sequenced and all three ITLNs have been shown to upregulate in response to nematode infections in sheep and lambs. No functional studies have been undertaken in this thesis and further work is required to identify the role of intelectins and sialyltransferases in a Th2 environment. ITLNs and SIAT4C may have important roles to play in nematode infections in sheep.

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PUBLICATIONS ARISING FROM THIS THESIS

PAPERS

1. French, A.T., Bethune, J.A., Knight, P.A., McNeilly, T.N.,Wattegedera, S., Rhind, S., Miller, H.R.P., Pemberton, A.D. 2007. The expression of intelectin in sheep goblet cells and upregulation by interleukin-4. Veterinary Immunology and Immunopathology 120, 41–46.

2. French, A.T., Knight, P.A., Smith, W.D., Brown, J., Craig, N.M., Pate, J.A., Miller, H.R.P., Pemberton A.D. 2008. Up-regulation of intelectin in sheep after infection with Teladorsagia circumcincta International Journal for Parasitology 38 467–475.

ABSTRACTS

1. French, A.T., Knight, P.A., Smith, W.D., Collie, D.D., Pate, J., Miller, H.R.P., Pemberton, A.D. 2007. Upregulation of intelectin expression in parasitic infections in the gut and respiratory tract of sheep. Proceedings of the 61st AVTRW Annual congress, Scarborough, UK

2. French, A.T., Knight, P.A., Smith, W.D., Pate, J., Miller, H.R.P., Pemberton, A.D. 2006. Upregulation of Intelectin expression in *Teladorsagia circumcincta* infection in sheep. Proceedings of ICOPA XI, Glasgow, UK

3. French, A.T., Pemberton, A.D., Wright, S.H., Smith, W.D., Knight, P.A., Pate, J., Finney, C.A.M., Miller, H.R.P. 2006. Upregulation of intelectin expression in T helper type (Th) 2 responses in the gut and respiratory tract mucosa. Proceedings of Keystone Symposium; Asthma and allergic inflammation, Colorado, USA.

4. French, A.T., Pemberton, A.D., Wright, S.H., Smith, W.D., Entrican, G., Bethune, J., Knight, P.A., Pate, J., Miller, H.R.P. 2006. Upregulation of intelectin expression in T helper type (Th) 2 responses in the gut and respiratory tract mucosa. Proceedings of the 60TH AVTRW Annual congress, Scarborough, UK.

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The expression of intelectin in sheep goblet cells and upregulation by interleukin-4☆

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Abstract

Upregulation of intelectin (ITLN) transcript and protein has previously been shown in intestinal nematode infections of resistant mice strains with immunolocalisation of protein to goblet cells and paneth cells. In man, intelectin expression has been shown in respiratory tract epithelium, with upregulation occurring in bronchoalveolar lavage fluid of asthmatic individuals. This study describes the expression of intelectin in the respiratory tract of sheep and the immunolocalisation to goblet cells using a novel affinity-purified chicken anti-intelectin peptide antibody. Furthermore we show that when sheep tracheal explants were cultured for 48 h± recombinant sheep IL-4, sheep ITLN transcripts were upregulated compared with controls. Putative roles for intelectin have included an antibacterial role and an alteration of the character of mucus. Our data suggest ITLNs may play an important role in the mucosal response in allergy and parasitic infections.

Keywords: Sheep; Intelectin; Goblet cell; IL-4; Th2 response

1. Introduction

The intelectin (ITLN) family is composed of calcium-dependent galactose binding lectins with homologues known in sea squirts, fishes, frogs and mammals (Chang et al., 2004). The first mammalian intelectin to be described was mouse intelectin-1 in 1998 (Komiya et al., 1998), which was shown to be expressed by small intestinal paneth cells and a role in defence against bacterial infection was suggested.

Human intelectin-1 was subsequently described by separate groups (Lee et al., 2001; Tsuji et al., 2001) and recombinant intelectin-1 was found to bind galactofuranose residues isolated from the bacterium *Nocardia* as well as ribose and deoxyribose, and to a lesser extent, galactose (Tsuji et al., 2001) lending further support for a role of intelectin in innate recognition of bacteria.

Human intelectin-1, also known as human lactoferrin receptor, binds lactoferrin which has anti-microbial, immunomodulatory and anti-inflammatory properties (Suzuki et al., 2001). More recently it has been recognised as omentin and has been isolated from omental fat of patients with inflammatory diseases such as ulcerative colitis (Schaffler et al., 2005) and shown to enhance insulin-mediated uptake of glucose in adipocytes (Yang et al., 2006).

☆ Mouse intelectin-1 and intelectin-2 are not direct orthologues of human intelectin-1 and intelectin-2, respectively. However, this nomenclature is used for consistency with previous literature. Synonyms for mouse intelectin-1 are intelectin, intelectin-a, intelectin-1a and lactoferrin receptor. Synonyms for mouse intelectin-2 are intelectin-b and intelectin-1b.

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Additionally, a novel intelectin variant, mouse intelectin-2, was found to be highly upregulated in intestinal epithelium from the resistant BALB/c mouse strain following infection with *Trichinella spiralis* (Pemberton et al., 2004a). Results were confirmed by RT-PCR and intelectin was immunolocalised to the paneth cells and goblet cells (Pemberton et al., 2004a). There was differential expression of mouse ITLN1 and 2, with constitutive expression of mITLN1 which did not show upregulation on infection. In a gene profiling analysis, significant upregulation of intelectin expression was shown in the caecum of resistant mice infected with *Trichuris muris* (Datta et al., 2005). Subsequently, Artis (2006) confirmed upregulation of ITLN1 and 2 by RT-PCR in this model, and once again intelectin was immunolocalised to goblet cells. In both these models of nematode infection, differential expression of intestinal intelectins has been shown in susceptible and resistant strains of mice, and a potential anti-nematode role has been suggested (Artis, 2006; Pemberton et al., 2004a).

Allergic airway sensitisation in asthmatics, as well as enteric responses to gastrointestinal nematodes, share similarities in that both involve polarisation of T cells and expression of the Th2 cytokines, IL-4 and IL-13 (Datta et al., 2005; Walter and Holtzman, 2005). It is thus of interest that Kuperman et al. (2005) have shown an increase in intelectin expression in mice over expressing IL-13. Furthermore, intelectin has been also shown to be upregulated in bronchial brushings (Kuperman et al., 2005) and bronchoalveolar lavage fluid (Wu et al., 2005) from asthmatics. We now show expression of intelectin in sheep airways, a common model for asthma in man (Abraham et al., 2005; Bischof et al., 2003) and upregulation of ITLN transcription in sheep tracheal explants by recombinant sheep IL-4.

2. Materials and methods

2.1. Antibody preparation and validation

2.1.1. Peptide for commercial antibody preparation

A peptide was chosen for commercial antibody preparation in chickens (Aves Labs, Tigard, OR, USA), based on predicted immunogenicity and high degree of sequence conservation between species, ITLN peptide: TSDDYKNPGY(F/Y)DIQA, corresponding to residues 130–143 of mITLN2. Specific anti-peptide IgY was purified from total IgY by affinity chromatography, using the appropriate peptide immobilised on NHS-activated sepharose (Hi-Trap NHS activated, 1 ml, G.E. Healthcare).

2.1.2. Tissues for validation of antibody

2.1.2.1. Mouse, human and sheep. Mouse: BALB/c mice were chosen as they are known to upregulate jejunal intelectin expression during *T. spiralis* infection (Pemberton et al., 2004a). For *T. spiralis* infection 8–15-week-old BALB/c (B & K Universal, Hull, UK) were infected by gavages with 200–300 muscle larvae per mouse in 0.2 ml of phosphate buffered saline (PBS)/0.1% agar. To check infections were successful, adult worms were isolated from groups of four to five of the mice at 6–7 days after infection. For protein extraction mice were killed 14 days after infection, and jejunum was snap frozen and stored at −70 °C. Jejunal sections were fixed in 4% paraformaldehyde for immunohistochemistry. All experiments were approved by the University of Edinburgh's Biological Services ethical review committee and were performed under license, as required by the United Kingdom's Animals (Scientific Procedures) Act of 1986. Mice were maintained in a conventional environment at the University of Edinburgh.

Human: We have found that LS174T cells, a human colonic mucoid adenocarcinoma cell line, upregulates human intelectin-1 in response to the cytokines IL-4 and IL-13 (manuscript in preparation). Thus LS174T cells (European collection of Animal Cell Cultures, Porton Down, UK) were cultured in Eagle's minimum essential medium (Invitrogen, Paisley, UK) supplemented with non-essential amino acids, 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2 mmol L-glutamine (Invitrogen) and 10% heat inactivated foetal calf serum (Invitrogen) at 37 °C with 5% CO_2. Recombinant human IL-4 (Peprotech EC, London, UK) was made up according to the manufacturer's recommendations and was used at a concentration of 1 ng/ml. Cells were harvested at 72 h, the supernatant was discarded and cells stored at −70 °C for protein extraction.

Sheep: Tracheal mucosa was stripped from freshly culled aged ewes and stored at −70 °C for protein extraction. Trachea and lung sections were fixed in 4% paraformaldehyde for immunohistochemistry.

2.1.3. Western blot

Protein was extracted from jejunum of *T. spiralis*-infected BALB/c mice, IL-4 treated LS174T cells and sheep tracheal epithelium, and run on 12% acrylamide SDS-PAGE mini gels (mini-Protean, Bio-Rad). The gels were blotted (Transblot SD, Bio-Rad) onto polyvinylidenefluoride (PVDF) membrane (Immobilon P, Millipore) and blocked with 50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 1% milk powder, 0.5% Tween 80. Blots

were incubated for 1 h with 1 μg/ml of either affinity-purified chicken polyclonal antibody to ITLN peptide or control chicken IgY in blocking buffer. Following washing, membranes were incubated for 1 h with the horseradish peroxidase conjugated rabbit anti-chicken IgY (Sigma, A9046), at a dilution of 1:10,000. After a further wash, colour was developed with 3,3′-diaminobenzidine (Vector laboratories, Burlingame, USA).

2.2. IL-4 treatment of isolated sheep tracheal explants

2.2.1. Culture of explants

Tracheal explants were harvested from five freshly culled aged ewes. The culture technique was a modification of previously published techniques (Kitson et al., 1999; Lin et al., 2001). Tracheal rings were harvested, washed three times in PBS containing 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml amphotericin B and 10 μg/ml gentamycin and excess fat was trimmed. Equal sized discs of tracheal mucosa were dissected from the underlying cartilage using a 6 mm sterile punch biopsy (Kruuse, A/S, Marsley, Denmark) and placed epithelial surface upwards into the bottom of 6.5 mm diameter, 0.4 μm pore size Transwell® cell culture inserts (Corning Life Sciences, Acton, USA) within 24-well tissue culture plates. Mucosal explants were cultured in an air–liquid interface for 48 h at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) containing 2% ultraserosa G serum substitute (Ciphergen, Fremont, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml amphotericin, 10 μg/ml gentamycin and 100 U/ml insulin with ($n = 3$) or without ($n = 3$) recombinant sheep IL-4 at a 1:100 dilution. Recombinant sheep IL-4 was expressed in Chinese hamster ovary (CHO) cells as previously described (Hope et al., 2005), and the resulting supernatant and control CHO cell supernatant were used at 1:100 dilution. Explants were harvested and fixed in 4% paraformaldehyde for immunohistochemistry or placed in RNA later (Qiagen) for RNA extraction.

2.2.2. RNA extraction and real-time RT-PCR of tracheal explants

Tissue was homogenised for RNA extraction using the Fastprep system (Obiogene, Cambridge, UK) and Qiashredder (Qiagen, Hilden, Germany), total RNA was extracted using the Mini RNeasy kit (Qiagen) as per the manufacturer's instructions. RNA was reverse transcribed using a Promega RT kit (Promega). Quantitative real-time PCR was performed using an Opticon 2 real-time PCR system (MJ research, GRI, Rayne, UK) and Quantitect SYBR Green (Qiagen) as the fluorescent probe. The intelectin PCR primers were as follows: ITLN forward 1 (F1): CAGAAGCTGCAAGGAAATCAA; ITLN reverse 1 (R1): AGCCAGGGTTCTTGTAGTCA. The consensus (human and mouse ITLN) primer ITLN F1 had previously been used to amplify a partial sequence of sheep intelectin (Accession No. AM087961) when used with the consensus primer ITLN R2; TTGTCAGTCCAACACTTTCCT. The primer ITLN R1 was identified in the partial sheep sequence and used with ITLN F1 to amplify a sheep ITLN product of 302 bp for real-time PCR. Sheep ATPase (ATPase catalytic subunit alpha; genbank accession no. X02813) was used as the house keeping gene, primer details were as follows: sheep ATPase forward, GCTGACTTGGTCATCTGC; sheep ATPase reverse, CAGGTAGGTTTGAGGGGATAC (PCR product 167 bp). The PCR conditions were 95 °C for 15 min, then 49 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Cycle thresholds (Ct) were normalized to the housekeeping gene sheep ATPase using ΔΔcT analysis, for quantification (Livak and Schmittgen, 2001). Reactions were carried out in triplicate and fluorescence values calculated against background and 'master mix' only controls using Opticon 2 software with subsequent analysis in Microsoft Excel and InStat (GraphPad Software).

2.3. Anti-ITLN immunohistochemistry and histochemistry

Tissues fixed in 4% paraformaldehyde were processed into paraffin blocks and sections (4 μm) were made onto coated slides (Snowcoat X-tra; Surgipath, Winnipeg, Manitoba, Canada). Sections were dewaxed in xylene, dehydrated through graded alcohols and endogenous peroxidase activity blocked by treatment with methanol containing 3% (v/v) of 30% hydrogen peroxide. After dehydration, sections were placed in 10 mM citric acid buffer pH 6.0 (mouse tissue) or 10 mM Tris–HCl pH 9.0 (sheep tissue) in a pressure cooker and brought to pressure in a microwave and held at pressure for 4 min. Slides were washed, blocked with PBS containing 10% normal rabbit serum 0.5 M NaCl and 0.5% Tween 80, and incubated with affinity-purified chicken anti-ITLN peptide (1 μg/ml in blocking buffer) or control chicken IgY (1 μg/ml in blocking buffer) either overnight (mouse tissue) or for 1 h (sheep tissue) at 21 °C. After treatment with horseradish peroxidase conjugated rabbit anti-chicken IgY (Sigma, 1/500), sections were stained with Nova Red (Vector

Laboratories) counterstained with haematoxylin, then dehydrated and mounted. Paraformaldehyde fixed sections were stained with Alcian Blue/periodic acid Schiff for goblet cell detection and with haematoxylin and eosin to examine tissue viability.

3. Results and discussion

3.1.1. Antibodies against intelectins

Intelectin expression in the mouse was originally assessed using a rabbit polyclonal antibody against the frog egg lectin XL35 (Pemberton et al., 2004a). Since the intelectin epitopes identified with this antibody were not defined, an antibody was raised in chickens against a predicted surface loop peptide. The anti-ITLN peptide antibody recognised intelectins when used to probe blots of preparations containing human goblet cell intelectin, mouse intestinal intelectin and sheep tracheal intelectin (Fig. 1). The specificity of the affinity-purified antibody was tested also by immunohistochemistry in mouse intestinal tract and sheep respiratory tract. It detected abundant goblet cells in *T. spiralis*-infected BALB/c mouse jejunum, in which mITLN2 is strongly upregulated (Pemberton et al., 2004b), as previously described with the rabbit anti-XL35 polyclonal antibody (Pemberton et al., 2004a). In addition, this antibody labelled paneth cells, which express mITLN1 (Komiya et al., 1998) but which were not labelled with anti-XL35. This suggests that the XL35 antibody does not detect mITLN1. Control IgY was negative. There was strong constitutive expression of intelectin in the goblet cells and weak expression in the submucosal glands of sheep respiratory tract (Fig. 2).

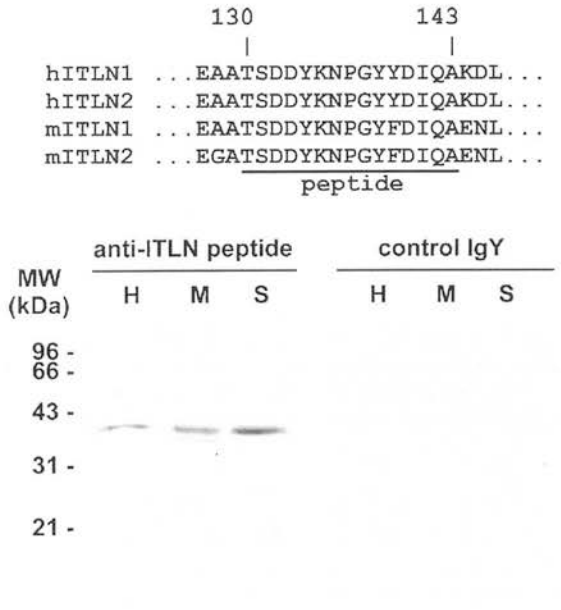

Fig. 1. Characterisation of anti-ITLN peptide. The region of the human and mouse intelectin sequence chosen for anti-peptide antiserum generation are indicated. The antibody-detected intelectins by Western blotting in samples of human (H; extract of IL-4 treated LS174T cells), mouse (M; extract of *Trichinella spiralis*-infected mouse jejunum) and sheep (S; extract of tracheal epithelium) origin, see Section 2 for details. Control IgY was negative.

3.2. Regulation of intelectin expression in sheep airways by IL-4

Because of the known increase in intelectin expression in asthmatic human subjects and in mouse models of asthma (Kuperman et al., 2005), which are both mediated by Th2 cytokines, we examined the effect of IL-4 on the level of intelectin expression in an ex vivo model, using sheep tracheal explants. Sheep were of particular interest because they have similar lung physiology to man, and there are several models of asthma in this species (Abraham et al., 2005; Bischof et al., 2003). Incubations with recombinant sheep IL-4 and control medium were carried out for 48 h, since preliminary studies showed that surface epithelium in the explants remained fully viable for the first 48 h of culture, but had degenerated by 72 h (not shown). Freshly isolated explants all expressed intelectin, and protein was detected by immunohistochemistry in the goblet cells. Abundant epithelial goblet cells were also demonstrated by Alcian Blue/PAS staining (Fig. 2). The levels of intelectin declined when the explants were cultured in the absence of exogenous cytokines, but with the addition of recombinant sheep IL-4, intelectin expression increased. Using real-time PCR, expression of sITLN was shown to be upregulated in tracheal explants from five out of five sheep cultured in medium containing recombinant sheep IL-4 compared to medium alone (Fig. 3); $p = 0.06$ using the non-parametric paired Wilcoxon signed rank test. Examination of H and E stained sections by a pathologist, blinded to the section identity, confirmed that there were no histologically evident differences in viability between the explants maintained in medium alone compared to medium containing sheep IL-4.

In summary, using the chicken anti-peptide antibodies, we have been able to demonstrate the presence of intelectin in sheep airways, apparently expressed constitutively and present both in the airway goblet cells and in the submucosal glands. This is the first study to show intelectin expression in the goblet cells of the

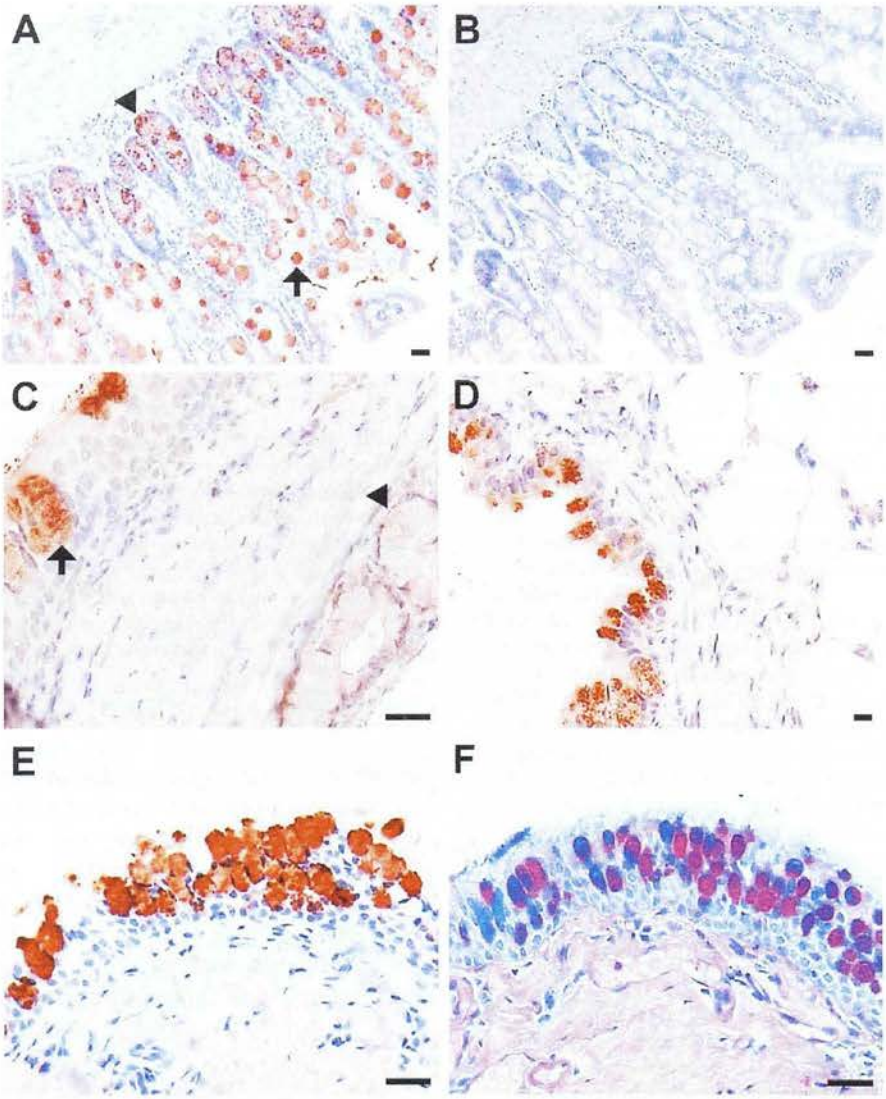

Fig. 2. Intelectin immunohistochemistry. Immunoperoxidase labelling of sections with affinity-purified chicken anti-intelectin peptide IgY (anti-ITLN peptide) was carried out as described in Section 2. Panels A and B show labelling of *T. spiralis*-infected BALB/c mouse jejunum (day 14 of infection) with anti-ITLN peptide and control IgY, respectively (paneth cells marked by arrow head, goblet cells by arrow). Panels C and D show sheep lung sections with strong immunolabelling of goblet cells and weak labelling of submucosal glands with anti-ITLN peptide (goblet cells marked by arrow, submucosal glands arrow head). Panels E and F show sheep tracheal explants labelled with anti-ITLN peptide and Alcian Blue/periodic acid Schiff reagent, respectively. These show abundant ITLN positive goblet cells in the tracheal epithelium.

respiratory tract mucosa. We also demonstrate for the first time that upregulation of intelectin is IL-4 dependent, providing further support to the differential/preferential expression of intelectins in response to Th2-mediated inflammation. Intelectins share homology with *Xenopus laevis* egg lectin, which alters the character of the egg jelly coat by cross-linking glycoproteins thus preventing polyspermy (Nishihara et al., 1986). It is thus possible that intelectins may play a role in altering the physical properties of mucus or alternatively they may be acting as a Th2-induced antiseptic paint that reduces bacterial colonisation where the normal Th1 reactivity has been suppressed. Further investigations into the expression of intelectin in respiratory and gastrointestinal nematode infections in sheep are underway. In the present era of increasing prevalence of asthma in man and increased anthelminthic resistance in sheep nematodes, intelectin is a very interesting new molecule which may offer prospects as a future drug target.

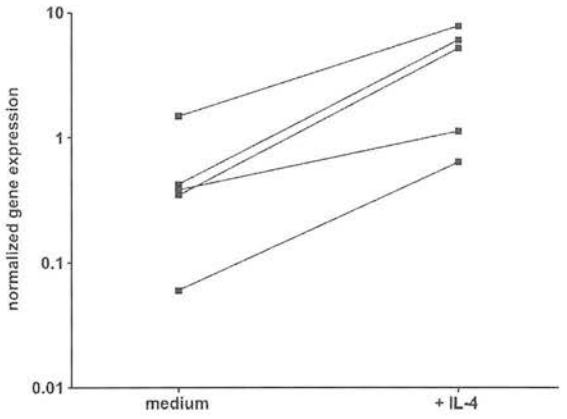

Fig. 3. Expression of sheep tracheal intelectin in response to recombinant sheep IL-4. The relative abundance of intelectin transcript in RNA isolated from tracheal explants of five individual sheep as quantified by real-time RT-PCR is shown. Each point represents the mean of three biological replicates from a single sheep, each of which was analysed in triplicate by real-time PCR. The mean expression of sheep intelectin mRNA was upregulated in tracheal explants from five out of five sheep incubated for 48 h with medium containing recombinant sheep IL-4 compared to explants incubated in medium alone.

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Up-regulation of intelectin in sheep after infection with *Teladorsagia circumcincta* ☆

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Abstract

A novel intelectin molecule designated sheep intelectin 2 (sITLN2) was detected in sheep abomasal mucosa. The full sequence shared 76–83% homology with other mammalian intelectins. Intelectins are mucus-associated proteins that have been shown to be up-regulated in gastrointestinal nematode infections in rodents and in human asthma. Expression of sheep abomasal ITLN2 mRNA was significantly up-regulated on day 10 post-challenge of worm-free sheep with *Teladorsagia circumcincta* and at day 2 in previously infected, immune sheep. Increased expression of ITLN protein following challenge was confirmed by Western blot and was immunolocalised to the mucous neck cells of the abomasal mucosa. Infection with *T. circumcincta* was also associated with increased levels of abomasal transcripts encoding sheep mast cell protease-1, ovine galectin-14 and IL4, which collectively suggested a Th2 type response. Intelectin may play an important role in the mucosal response to gastrointestinal nematode infections in ruminants.

Keywords: Intelectin; Abomasum; Sheep; *Teladorsagia circumcincta*; sMCP-1; OvGal-14; IL4

1. Introduction

Parasitic gastroenteritis is a major problem in sheep farming worldwide causing significant production loss. Whilst the genera *Haemonchus, Ostertagia, Teladorsagia* and *Trichostrongylus* spp. can all contribute significantly to this disease syndrome, the abomasal parasite, *Teladorsagia circumcincta* has been shown to play a major role in temperate climates. Immunity can develop on repeated exposure to *T. circumcincta* (Smith et al., 1983a,b) and appears to be related to age (Smith et al., 1985) and genetic background (Stear et al., 1999; Davies et al., 2005). Whilst eosinophils (Wildblood et al., 2005; Henderson and Stear, 2006), mast cells/globule leukocytes (Miller, 1996; Huntley et al., 2004), IgA (Stear et al., 2004; Martinez-Valladares et al., 2005; Halliday et al., 2007), IgE (Huntley et al., 1998; Pettit et al., 2005) and mucus (Scott et al., 1998) have all been implicated, the main effectors of this acquired immunity still remain to be elucidated.

Control of gastrointestinal helminths in farm animals in recent years has relied heavily on the use of anthelmintics. However, increasing concerns regarding drug residues and emerging anthelmintic resistance (Wolstenholme et al., 2004; Coles, 2005; Wrigley et al., 2006) have encouraged investigation of alternative control strategies including vaccination (Knox and Smith, 2001; Redmond et al., 2006; Smith and Zarlenga, 2006), natural anthelmintics (Shaik et al., 2006; Athanasiadou et al., 2007), nematode destroying fungi (Mendoza et al., 2006) and selective breeding for host resistance (Davies et al., 2005). Our understanding of the immune response to gastro-intestinal helminth infections is important for the development of new immunological control strategies and recent studies using rodent

☆ *Note.* Nucleotide sequence data reported in this paper is available in the GenBank™ database under the Accession No. EF521881.

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models have highlighted the importance of the innate mucosal response in immunity.

We have recently identified a novel intelectin (ITLN), intelectin-2 (ITLN2), as an abundantly expressed jejunal epithelial protein in parasitised BALB/c mice (Pemberton et al., 2004b). Mouse intelectin-2 (mITLN2) is undetectable in uninfected controls, but is maximally expressed at the time of rejection of *Trichinella spiralis* and is present in both goblet cells and in the mucus layer. In contrast, with the massively up-regulated expression of mITLN2, transcription of the 95% identical Paneth cell-derived mITLN1 gene was constitutive and little altered in infected animals (Pemberton et al., 2004a). ITLNs appear to have arisen early in chordate evolution, and homologues are known in sea squirts, fishes (Chang and Nie, 2007; Gerwick et al., 2007), frogs (Chang et al., 2004) and mammals (Komiya et al., 1998; Lee et al., 2001; Suzuki et al., 2001). Mouse intelectin-1 (mITLN1) and mITLN2 are not direct orthologues of human intelectin-1 (hITLN1) and human intelectin-2 (hITLN2), respectively. However, this nomenclature is used for consistency with previous literature. Synonyms for mITLN1 are mouse intelectin, mouse intelectin a, mouse intelectin 1a and lactoferrin receptor. Synonyms for mITLN2 are mouse intelectin b and mouse intelectin 1b.

Whilst a putative anti-bacterial role has been suggested for hITLN1 (Tsuji et al., 2001), results of experimental work in mice suggest ITLN may represent an important novel effector gene involved in parasite rejection from the gastrointestinal tract (Pemberton et al., 2004a; Datta et al., 2005). However, a recent report using *Nippostrongylus brasiliensis* infection in mice has contradicted this suggestion (Voehringer et al., 2007).

In the present study, we have cloned and sequenced sheep abomasal ITLN (GenBank Accession No. EF521881) and investigated transcript and protein expression in the abomasal mucosa of sheep infected with *T. circumcincta*. Our results show that this ITLN transcript and protein are up-regulated following challenge infection and furthermore that expression is greater and occurs at an earlier time point following challenge in previously infected immune animals compared with naïve. We also show up-regulation of IL4, sheep mast cell protease-1 (sMCP-1) and ovine galectin-14 (OvGal-14) in the abomasal mucosa following challenge infection, confirming that intelectin up-regulation occurs in association with a Th2 type response to this abomasal parasite.

2. Materials and methods

2.1. Animals and parasites

2.1.1. Sheep

Scottish Blackface-cross yearling sheep were born and maintained at the Moredun Research Institute under conditions designed to exclude accidental infection with nematode parasites. All experiments were approved by the Institute's ethical review committee and were performed under licence, as required by the United Kingdom's Animals (Scientific Procedures) Act of 1986. Experiments were undertaken in 2005 and 2006.

2.1.2. Infective larvae

Larvae from an anthelmintic susceptible isolate of *T. circumcincta* (Moredun Research Institute) were isolated as previously described (Smith et al., 1983b) and stored at 4 °C for up to 1 month before use. All the challenge doses used within each experiment were from the same batch of larvae.

2.2. Parasite infections

Three similar experiments were conducted in successive years, the designs of which are summarised in Table 1. Sheep were divided into four groups; unchallenged naïve (unv), challenged naïve (cnv), unchallenged previously infected (upi) and challenged previously infected (cpi). The groups allowed comparisons to be made between the following two experimental treatments: naïve sheep responding to a primary infection with 50,000 *T. circumcincta* L3 and previously infected sheep responding to the same dose as a challenge infection. The previously infected

Table 1
Experimental design of three trials in which previously infected or naïve sheep were exposed to infection with *Teladorsagia circumcincta*

Trial (day)	Group	Treatments (relative to day of challenge)							
		Trickle[a] infection	Levamisole treatment[b]	Challenge[c] infection	Number killed				
			(−7)	(0)	(0)	(2)	(5)	(10)	(21)
1	Previously infected	+	+	+				6	
	Naïve	−	+	+				6	3
2	Previously infected	+	+	+			6	6	
	Naïve	−	+	+	6		6	6	6
3	Previously infected	+	+	+	6	6			
	Naive	−	+	+		6			

[a] Two-thousand *T. circumcincta* L3 3× per week for 8 weeks.
[b] Dose rate of levamisole 7.5 mg/kg.
[c] 1 × 50,000 *T. circumcincta* L3.

sheep were given 2000 L3 three times per week for 8 weeks, a protocol which has previously been shown to confer some immunity (Smith et al., 1983b). All sheep were treated with anthelmintic prior to challenge to clear any residual worms in the previously infected group, and as a control treatment in the other groups. The serial kill provided limited information on the kinetics of the responses in both challenged groups.

2.3. Post-mortem procedure

The sheep were stunned with a captive bolt and exsanguinated. The abomasum was removed, contents collected and then opened longitudinally. One half of the abomasum was retained for worm counting along with the abomasal contents as described previously (Halliday et al., 2007). Abomasal folds from the other half of the fundic region were used for tissue sample collection. Tissue samples were stored in RNA later (Qiagen, Hilden, Germany) for RNA isolation, snap frozen for protein extraction or fixed in 4% paraformaldehyde for immunohistochemistry.

2.4. Sequencing of sheep intelectin

Abomasal tissue from *T. circumcincta*-infected sheep was homogenised using a Stratech Beadbeater-B (Stratech Scientific, Scham, UK) with 1 mm^3 Zirconia/silica beads (Thistle Scientific, Glasgow, Scotland, UK) and filtered through a Qiashredder (Qiagen, Crawley, UK). Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed using a Promega RT kit (Promega, Southampton, UK). Consensus primers for conserved regions of human and mouse intelectins (cITLN F1 and cITLN R2) were used to amplify a partial ITLN sequence, which on sequencing was found to share 91% homology with hITLN1. Internal (Int) and external (Ext) gene-specific primers (GSP) were designed based on this partial sequence for use with the BD smart race cDNA amplification kit (BD Biosciences, Clontech, California, USA). 5′ and 3′ rapid amplification of cDNA (RACE) ready cDNA was prepared using BD SMART II™ A Oligonucleotide and the BD PowerScript™ Reverse Transcriptase (RT) according to the manufacturer's recommendations. 5′ and 3′ RACE ready cDNA was then amplified using the supplied universal primer A mix with the primers sITLN Ext GSP 2 and sITLN Ext GSP 1 for the 5′ and 3′ ends, respectively, and BD advantage polymerase (BD Biosciences, Clontech). The 5′ end initial amplification was followed by a nested amplification using sITLN Int GSP2, nested universal primer A, 0.5 m GC melt (BD Biosciences, Clontech) and BD Advantage™ GC 2 Polymerase (BD Biosciences, Clontech). For both the initial and the nested reaction the conditions were the same: 35 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 3 min. For amplification of the 3′ end, touch down PCR was used, conditions were as follows: five cycles of 94 °C for 30 min, 72 °C for 3 min; five cycles of 94 °C for 30 min, 70 °C for 30 s, 72 °C for 3 min; 32 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 3 min. Resultant PCR products were visualised on ethidium bromide-stained 1.4% agarose gels and images recorded using Kodak Digital Science ImageStation 440CF and analysed using 1D Image Analysis software. Primer sequences are given in Table 2.

Table 2
Primers used in sequencing, semi-quantitative and quantitative PCR

Primer name	Primer sequence 5′–3′		Product size (bp)	Annealing temperature (°C)
Consensus ITLN F1	Fw[a]	^{265}CAGAAGCTGCAAGGAAATCAA^{286}	496	58
Consensus ITLN R2	Rv[b]	^{760}TTGTCAGTCCAACACTTTCCT^{740}		
Sheep ITLN ext GSP 2	Fw	^{378}GGTGGCGGCTGGACCCTGGTG^{398}	309	65
Sheep ITLN ext GSP 1	Rv	^{686}GCTCCGGAAGAAGCCCGTGTTGG^{664}		
Sheep ITLN int GSP 2	Fw	^{434}CACGGTGGGCGATCGCTGGTC^{454}	176	65/60/58
Sheep ITLN int GSP 1	Rv	^{609}GCACGTGCCAGATGCCCAGGTC^{588}		
Sheep ITLN F1	Fw	^{6}GCTCTGAGACTGCTCCTGGT^{25}	1179	55
Sheep ITLN R1	Rv	^{1185}TTCTCCTTTGCTTGTTGGAGA^{1164}		
Sheep ATPase	Fw	GCTGACTTGGTCATCTGC	167	60
	Rv	CAGGTAGGTTTGAGGGGATAC		
Sheep IL4	Fw	AACGCCGAACATCCTCACAT	171	58
	Rv	AGTCCGCCCAGGAATTTG TT		
Sheep galectin-14	Fw	ATTCCTGTTGCAGAAGTCTACCTGGACA	545	61
	Rv	GAACATCTTCCACACGGTAGGGGT		
Sheep mast cell protease-1	Fw	ACATCGTGGACAGAGAGAGG	354	60
	Rv	TCTTCCTCTTGGTTGAATCTC		
Universal primer A long		CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT		
Universal primer A short		CTAATACGACTCACTATAGGGC		
Nested universal primer A		AAGCAGTGGTATCAACGCAGAGT		

ITLN, intelectin.
Superscript numbers refer to position in sequence (GenBank Accession No. EF521881).
[a] Forward.
[b] Reverse.

PCR products were cloned into the TA Cloning vector pCR2.1 (Invitrogen, Leek, The Netherlands). Positive clones were identified by restriction enzyme digestion with EcoRI (Roche) to excise the insert. All nucleotide sequences were determined using dideoxy chain termination cycle sequencing by the Functional Genomics Unit, Moredun Research Institute, Midlothian, UK.

Using the overlapping 5′ and 3′ sequences the full 1232 bp sheep abomasal ITLN sequence was determined. The full sequence was amplified from abomasal mucosa of five sheep using the primers sITLN F1 and sITLN R1. The resultant PCR products were purified (High Pure PCR Product Purification kit; Roche, Mannheim, Germany), the sequences verified, the predicted open-reading frame determined, and the predicted signal peptide and *N*-glycosylation sites identified.

2.5. Detection of transcripts by semi-quantitative RT-PCR

cDNA of abomasal mucosa was prepared as detailed above. cDNA (50 ng) was amplified by PCR with primers (Table 2) for sheep abomasal ITLN, sheep IL4, sheep mast cell protease-1, sheep galectin-14 and sheep ATPase using equivalent quantities of non-reversed transcribed RNA as negative controls. Reaction conditions were optimised to ensure that the number of thermocycles correlated with the amplification stage of the PCR. Reaction conditions were as follows: sITLN int GSP, 23 cycles of 94 °C for 40 s, 58 °C for 40 s and 72 °C for 2 min; sheep IL4, 40 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 2 min; sMCP-1, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; sheep galectin-14, 32 cycles of 94 °C for 40 s, 61 °C for 20 s, 72 °C for 2 min; sheep ATPase, 31 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The expected product sizes and sequences are shown in Table 2. Resultant PCR products were visualised on ethidium bromide-stained 1.6% agarose gels and images recorded using Kodak Digital Science ImageStation 440CF or Bio-Rad FX imager and analysed using 1D Image Analysis software.

2.6. Detection of ITLN transcript by quantitative real-time PCR

Quantitative RT-PCR was performed using an Opticon 1 real-time PCR machine (MJ Research, GRI, Rayne, UK) and Quantitect SYBR Green (Qiagen) as the fluorescent probe. The primers used were sITLN int GSP2 and sITLN int GSP1 (see Table 2). In order to generate a standard curve, a PCR product containing the target sequence was amplified using the primers sITLN ext GSP2 and sITLN ext GSP1 (sequence length 309 bp) (Table 2). Six different concentrations of the PCR product were used to generate the standard curve ensuring the target fell within the range tested. Two hundred ng of cDNA was amplified using 0.3 μM primers in 20 μl Sybr green mastermix (2.5 mM $MgCl_2$) (Qiagen). PCR conditions were as follows: 95 °C for 15 min, then 50 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Reactions were carried out in triplicate. Melting curves were calculated for each reaction to confirm the identity of the PCR product and to ensure primer–dimer formation did not occur. Master mix only controls were included in all reactions as blanks to check for contamination. Cycle thresholds were calculated using Opticon monitor software version 2.1 correcting fluorescent values against blanks. Copy number was calculated using the following formula: Y molecules/μl = (X g/μl DNA/$(330 \times 660)) \times 6.022 \times 10^{23}$. Subsequent analysis was carried out using Microsoft Excel 2003 and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA).

2.7. Detection of ITLN and beta-actin protein by Western blot

Abomasal tissue was homogenised using a Stratech Beadbeater-B (Stratech) with 1 mm^3 zirconia/silica beads (Thistle Scientific). A urea buffer was used for protein extraction: urea ultra (Sigma), 2% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate hydate (CHAPS, Sigma), 0.4% dithiothetol (Sigma) and protease inhibitors (complete mini, EDTA-free, Roche). Fifty micrograms of protein was run on 10% acrylamide SDS–PAGE mini gels (mini-Protean, Bio-Rad, California, USA). Having checked equal loading of proteins, gels were blotted (Transblot SD, Bio-Rad) onto polyvinylidenefluoride (PVDF) membrane (Immobilon P, Millipore) and blocked with 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 2% milk powder, 0.5% Tween 80. Blots were incubated overnight at 4 °C with both affinity-purified chicken polyclonal antibody to ITLN peptide (French et al., 2007; 0.45 μg/ml) and mouse antibody to beta-actin (Sigma Clone, AC 15; 1:10,000) in blocking buffer. Following washing, membranes were incubated for 1 h with goat anti-mouse alkaline phosphatase antibody 1:20,000 (Jackson ImmunoResearch), washed again and incubated for a further hour with the horseradish peroxidase-conjugated rabbit anti-chicken IgY (Sigma, A9046), at a dilution of 1:10,000. ITLN-specific labelling was visualised using chemilumescent peroxidase substrate (Sigma) and after washing, beta-actin-specific labelling was visualised using the chemiluminescence substrate (CPD) – Star (Boehringer-Mannheim, Germany). Images were acquired using the Kodak Digital Science ImageStation 440CF.

2.8. Anti-ITLN immunohistochemistry and histochemistry

Tissues fixed in 4% paraformaldehyde were processed into paraffin blocks and sections (4 μm) were placed onto coated slides (Snowcoat X-tra; Surgipath, Winnipeg, Manitoba, Canada). Sections were dewaxed in xylene, rehydrated through graded alcohols and endogenous peroxidase activity was blocked by treatment with methanol containing 3% v/v of 30% hydrogen peroxide (Sigma). After rehydration, sections were placed in 10 mM Tris–HCl, pH 9.0, in a pressure cooker, brought to pressure in

a microwave and held at pressure for 4 min. Slides were washed, blocked with PBS containing 10% normal rabbit serum, 0.5 M NaCl and 0.5% Tween 80, and incubated with affinity-purified chicken anti-intelectin (anti-ITLN) peptide (1 μg/ml in blocking buffer) or control chicken IgY (1 μg/ml in blocking buffer) for 1 h at 21 °C. After treatment with horseradish peroxidase-conjugated rabbit anti-chicken IgY (Sigma, 1/500), sections were stained with Nova Red (Vector Laboratories) counterstained with hematoxylin, then dehydrated and mounted.

2.9. Statistical analysis of semi-quantitative, quantitative PCR transcripts and intelectin protein

The Kruskal–Wallis test was used to determine if there were significant differences between groups and the Mann–Whitney test was used for all paired comparisons; P-values <0.05 were considered significant.

3. Results

3.1. Cloning and sequencing of sheep abomasal intelectin

The consensus primers, cITLN F1 and cITLN R2, based on known human and mouse ITLN sequences, resulted in amplification of a sheep abomasal partial sequence that shared 90% homology with hITLN1 (GenBank Accession No. NM017625) and 94% homology with the sheep respiratory tract ITLN partial sequence (GenBank Accession No. AM087961). Further abomasal ITLN sequence information was obtained using the BD smart race cDNA amplification kit (BD Biosciences, Clontech) as described above. The full sequence (1232 bp) thus obtained had a predicted open-reading frame of 972 bp and deduced amino acid sequence of 323 amino acids. Using primers sITLN F1 and sITLN R1, the full sequence was confirmed in RNA isolated from abomasal mucosal samples of five sheep and was found to be identical.

The deduced amino acid sequence of sheep abomasal ITLN is shown in Fig. 1 aligned with hITLN1 with which it shares 79% homology. As in hITLN1, a predicted signal peptide was identified in the sITLN, suggestive of the secretory nature of this molecule. Two predicted N-glycosylation sites were identified in the sheep sequence in contrast to one in hITLN1. To distinguish this sequence from the partial sheep respiratory sequence already lodged with GenBank, we designated this sITLN2.

3.2. Parasitology

Worm counts are not included in this paper as they have been published elsewhere (Halliday et al., 2007). In brief, the worm counts from the equivalent groups were not significantly different between trials ($P > 0.05$) and thus data were combined where replicates were present. Significantly fewer worms were found in the cpi sheep compared with the cnv sheep ($P < 0.01$). In addition, the cpi sheep contained a significantly higher proportion of arrested early L4s than the primary infection and these were significantly stunted.

3.3. Expression of intelectin, sMCP-1, OvGal-14 and IL4 transcripts in sheep abomasum after infection with T. circumcincta

The expression of the following transcripts was determined as indicators of the abomasal immune response to *T. circumcinta*; OvGal-14, a known marker of eosinophils (Dunphy et al., 2002), IL4, a Th2 cytokine and sMCP-1, a marker of mucosal mast cells (Pemberton et al., 2000).

```
sITLN    MPAEGTGVRFYLLLFLSVATRGPRAAGTLSVEKSWEEEIRAPYLSFLPRSCKEIKERCHK 60
hITLN1   ------MNQLSFLLFLIATTRG----WSTDEANTYFKEWTCSSSPSLPRSCKEIKDECPS 50
               :: :**** .:***     : .  ::: :*  ..  . *********:.* .

sITLN    AGDGLYQLRTENGVVYQTFCDMTSGGGGWTLVASVHENNMRGKCTVGDRWSSQQGNRADY 120
hITLN1   AFDGLYFLRTENGVIYQTFCDMTSGGGGWTLVASVHENDMRGKCTVGDRWSSQQGSKAVY 110
         * **** *******:************************:****************.:* *

sITLN    PEGDGNWVNYNTFGSAEAATSDDYKNPGYYDIQARDLGIWHVPNKSPLQHWRNSSLLRYH 180
hITLN1   PEGDGNWANYNTFGSAEAATSDDYKNPGYYDIQAKDLGIWHVPNKSPMQHWRNSSLLRYR 170
         *******.****************************:************:***********:

sITLN    TNTGFFQSLGHNLFGLYQKYPVKYGAGNCLTDNGPSIPVDYDFGDAEKTASYYSPYCQGE 240
hITLN1   TDTGFLQTLGHNLFGIYQKYPVKYGEGKCWTDNGPVIPVVYDFGDAQKTASYYSPYGQRE 230
         *:***:*:*******:********* *:* ***** *** ******:********* * *

sITLN    FVAGFVQFRVFNNERGANALCAGVRVTGCNTEHHCIGGGGFFPEGNPSQCGDFSAFDWDG 300
hITLN1   FTAGFVQFRVFNNERAANALCAGMRVTGCNTEHHCIGGGGYFPEASPQQCGDFSGFDWSG 290
         *.**************.*******:****************:***..*.******.***.*

sITLN    YGTHQGYSSSREITEAAVLLFYR 323
hITLN1   YGTHVGYSSSREITEAAVLLFYR 313
         **** ******************
```

Fig. 1. Alignment of predicted open-reading frame of sheep abomasal intelectin (sITLN) with human intelectin 1 (hITLN 1). Predicted signal peptides are marked with dashed underlines. The peptide used for producing the chicken anti-intelectin antibody is marked by a solid underline whilst predicted N-glycosylation sites are shown by double underlines.

OvGal-14, IL4 and sMCP-1 all showed weak expression in abomasum from unv sheep and were significantly up-regulated following *T. circumcincta* challenge infection in naïve sheep at the following time points: OvGal-14: days 10 and 21, IL4: days 10 and 21 and sMCP-1: days 2, 10 and 21. This suggested a Th2 response (Fig. 2). In upi sheep, there was no significant increase in expression of OvGal-14 and IL4 transcripts compared with unv sheep, whilst sMCP-1 was found to be significantly up-regulated. When previously infected sheep were challenged, there was significant up-regulation of OvGal-14 and IL4 seen at days 2, 5 and 10 post-challenge whilst for sMCP-1 there was no significant change following challenge at any time point. ITLN transcript showed low, variable expression in the unv sheep but was significantly up-regulated at days 10 and 21 post-challenge in naïve sheep. There was no significant difference in expression of ITLN between unv and upi sheep. However, following challenge of previously infected sheep, up-regulation of ITLN transcript was seen at days 2, 5 and 10 (Fig. 2). Significantly ($P = 0.0036$) increased expression of sITLN at day 5 in cpi sheep compared with cnv sheep at the same time point was confirmed by quantitative real-time PCR (Fig. 3).

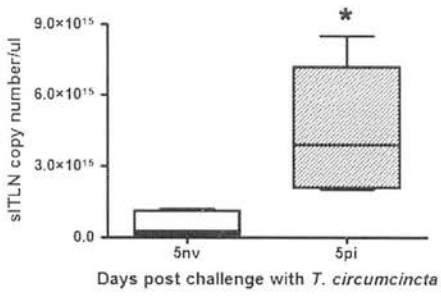

Fig. 3. Intelectin gene transcript copy number determined by quantitative real-time PCR in abomasal mucosa of challenged previously infected sheep and challenged naïve sheep at day 5 post-challenge with 50,000 *Teladorsagia circumcincta* larvae. Boxes represent the 25th and 75th quartiles; the median is shown within the box, the bars represent the range of the data. There was a significant (*) increase in expression of intelectin (ITLN) in the challenged previously infected sheep compared to the challenged naïve sheep at day 5 post-challenge ($P = 0.0036$).

3.4. Expression of intelectin protein in sheep abomasum after infection with *T. circumcincta*

The up-regulation of ITLN protein expression in infected sheep was confirmed by Western blot using the chicken anti-ITLN antibody (Fig. 4) in sheep from experiment 2. Low levels of protein expression were seen in three of the six unv sheep, whilst expression was not detected in the remaining three. Low levels of protein expression were detected in five of the six cnv sheep at day 5 following challenge whilst strong protein expression was present in all the cpi sheep at the same time point. Protein expression was seen in all cnv sheep at days 10 and 21 and in cpi sheep at day 10 post-challenge. Protein loading (not shown) was shown to be equal for all samples and beta-actin protein expression was found to be consistent in all samples (Fig. 4).

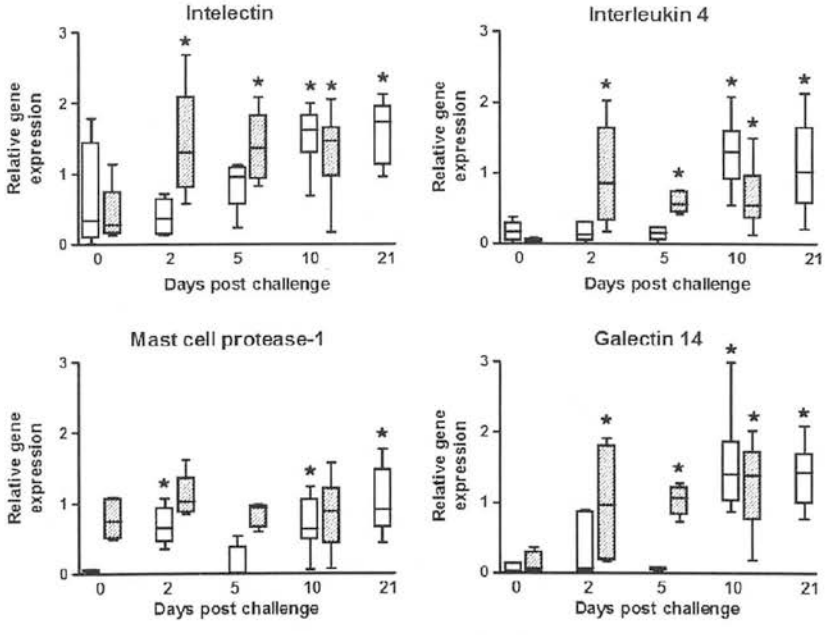

Fig. 2. Relative gene transcript expression by RT-PCR in abomasal mucosa of unchallenged naïve (unv), unchallenged previously infected (upi), *Teladorsagia circumcincta* challenged naïve (cnv) and *T. circumcincta* challenged previously infected (cpi) sheep (Table 1). The unv and cnv groups are shown as clear boxes, whilst the upi and cpi groups as hatched boxes; numbers of sheep examined is shown in Table 1. Data from different trials have been combined. Boxes represent the 25th and 75th quartiles; the median is shown within the box, the bars represent the range of the data. Statistical significance is shown by an asterisk (*).

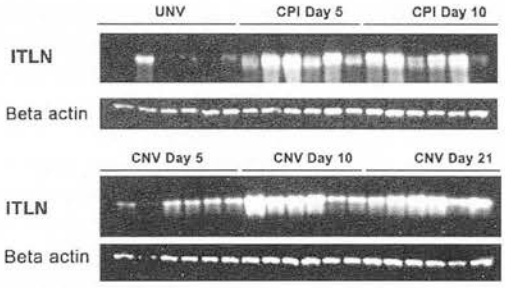

Fig. 4. Western blot showing expression of the 38 kDa sheep intelectin (ITLN) in abomasal mucosa of unchallenged naïve sheep (unv), challenged previously infected sheep (cpi) at days 5 and 10 and challenged naïve (cnv) sheep at days 5, 10 and 21 post-challenge with *Teladorsagia circumcincta*. For each group $n = 6$. Expression of beta-actin is shown as a control. Protein loading was equal (not shown).

3.5. Localisation of abomasal intelectin

ITLN protein expression was immunolocalised to abomasal mucus neck cells (Fig. 5) and was present at a much higher level in infected abomasal mucosa compared with that of worm-free sheep (Fig. 5B and A). It was notable that ITLN was detected in the gastric mucus surrounding the developing larvae and within the intestine of the parasites (Fig. 5C).

4. Discussion

We have previously demonstrated the expression of ITLN transcript and protein in the ovine respiratory tract (French et al., 2007) and now demonstrate that it is also expressed in the abomasum. The ITLN cloned and sequenced from sheep abomasum appears novel, with a predicted open-reading frame sharing 94% homology with that identified in sheep respiratory tract. Whilst the previously identified sheep respiratory ITLN transcript is constitutively expressed in normal sheep lung tissue (French et al., 2007), we have been unable to show expression of this novel ITLN transcript in normal sheep lung tissue (data not shown), suggesting that it represents a different ITLN. The antibody used for immunohistochemistry and Western blot was raised to a peptide sequence shared by both ITLNs and is thus non-selective. In vitro experiments have shown up-regulation of ITLN transcript in response to the Th2 cytokines IL4 and IL13 (Kuperman et al., 2005; French et al., 2007) and in vivo up-regulation has been seen with intestinal parasitic infections in mice and asthma in people (Pemberton et al., 2004a; Datta et al., 2005; Artis, 2006). Challenge infection of sheep with *T. circumcincta* has recently been shown to induce a Th2 cytokine profile bias in gastric lymph nodes (Craig et al., 2007) which is enhanced in previously infected sheep in comparison to naïve animals. Here, using primers specific for sITLN2 in the abomasum, we have shown up-regulation of ITLN transcripts within the abomasal mucosa of sheep challenged with *T. circumcincta* and the concurrent up-regulation of mast cell and eosinophil-specific transcripts sMCP-1 and OvGal-14, and the Th2-specific cytokine IL4. These data provide further supportive evidence of a Th2 response in the gastric mucosa following infection with *T. circumcincta*.

The expression of ITLN protein in five of six sheep as early as day 5 post-challenge of naïve animals is supportive of a role for ITLN in the early response to parasites. Of particular interest is the significant increase in ITLN transcript seen at a much earlier time point post-challenge in previously infected (immune) sheep compared with naïve sheep which may suggest a protective role for ITLN. Previous publications have shown the up-regulation/induction of ITLN transcript in response to infection with the gastro-intestinal nematodes *T. spiralis* and *Trichuris muris* in mice. Interestingly in *T. muris* infection in the intestine of BALB/c and AKR mice, up-regulation of ITLN was shown in the resistant BALB/c mouse strain but not in the susceptible AKR strain (Datta et al., 2005). These findings provide further evidence associating ITLN expression with the immune response to parasite infections.

It is possible that ITLN may play a role in altering the characteristics of mucus leading to worm entrapment. Previous studies have shown that on fertilisation the frog egg lectin (XL35; 60% identity with mammalian intelectins) interacts with mucin-like proteins to alter the characteristics of the egg coat protein and thus prevents polyspermy (Chang et al., 2004). Recombinant hITLN1 has been shown to recognise D-galactofuranosyl residues present in the arabinogalactan on the cell wall of *Nocardia rubra* (Tsuji et al., 2001) and thus ITLN may play a protective antibac-

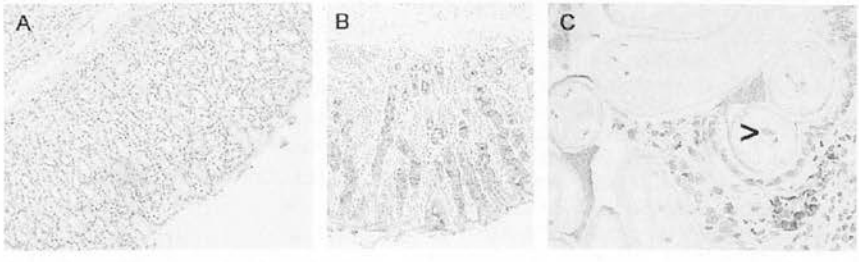

Fig. 5. Immunoperoxidase labelling of sections with affinity purified chicken anti-intelectin peptide IgY (anti-ITLN peptide). Sections are from an unchallenged naïve sheep (A), a challenged previously infected sheep 10 days post-challenge with *Teladorsagia circumcincta* (B), and a challenged naïve sheep 10 days post-challenge with *T. circumcincta* demonstrating intelectin positive mucus surrounding an L4 larva with positive staining of mucus in the lumen of the larval gut (C, arrowed). Control IgY labelling was negative (not shown).

terial role following mucosal damage by invading parasites. It is of interest that the homologous ascidian plasma lectin has been shown to increase phagocytic activity of *Halocynthia roretzi* hemocytes towards sheep red blood cells (Abe et al., 1999) and may support a role for ITLN in cell activation. In a transgenic mouse model, mITLN1 and mITLN2 have recently been shown to be part of the innate response to *Nippostrongylus braziliensis* infection. However overexpression of ITLN in the lung had no effect on parasite expulsion (Voehringer et al., 2007). It is unknown if this holds true for other nematode species in different hosts.

OvGal-14 has been shown to be a specific marker of eosinophils in sheep (Dunphy et al., 2002) and the results in this paper agree with previous findings of a marked eosinophilic infiltrate of the mucosa associated with *T. circumcincta* infection (Stear et al., 1995; Scott et al., 2000). OvGal-14 transcript expression peaked at day 10 post-challenge in naïve sheep in agreement with previous work where peripheral eosinophilia was shown to peak 8–10 days post-challenge infection in naïve sheep (Henderson and Stear, 2006). Whilst eosinophils have been associated with killing incoming larvae of *Haemonchus contortus* (Balic et al., 2006) no protective role has been shown to date in *T. circumcincta* infection. It is of interest that adult stages of *T. circumcincta* and excretory/secretory material from *T. circumcincta* L3s have been shown to produce potent chemo-attractant activity for bone marrow-derived eosinophils in vitro raising the question whether eosinophils may be permissive towards, rather than protective against, the parasites (Wildblood et al., 2005). In the present experiments the earlier increase in OvGal-14 transcript in previously infected sheep compared with naïve sheep may support a protective role for eosinophils.

The increased expression of sMCP-1 transcript after *T. circumcincta* infection agreed with previous findings of increased mast cell infiltration of the abomasal mucosa. Of particular interest was the very high level of transcript in upi sheep compared with unv sheep which may support a role in immunity. Previous work has shown an association between increased numbers of degranulated mast cells (globule leukocytes) in the mucosa and fewer nematodes in sheep (Seaton et al., 1989; Stear et al., 1995) and mast cells have been implicated in a hypersensitivity reaction in immune sheep resulting in rapid expulsion of gastro-intestinal parasites (Stear et al., 1995; Miller, 1996).

Infection with *T. circumcincta* is characterised by mucosal hyperplasia, and whilst a considerable number of studies have focused on the role of immunoglobulins, mast cells and eosinophils in natural immunity, very little work has focused on the role of mucus and the constituents of mucus. It is of interest that another lectin, ovine galectin-11, subsequently renamed galectin-15 (Gray et al., 2004), has been found in abundance in gastro-intestinal tract mucus following helminth infection and a role in altering the properties and activities of immune mucus has been suggested (Dunphy et al., 2000). In the highly glycosylated brush border/mucus interface, secreted lectins such as galectin-15 and ITLNs are ideally located to influence mucus properties. Intelectin is a novel mucus-associated molecule and further studies are required to elucidate whether it does play a protective role in parasitic infections in sheep and whether by altering the character of mucus it may be detrimental in allergic conditions.

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